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Pheromonal Interactions Among Cordate Gametophytes of the Lady Fern, *Athyrium filix-femina*

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ABSTRACT.—Pheromonal interactions between cordate gametophytes of the lady fern, *A. filix-femina,* were assayed using a protocol typically used for detecting water-soluble pheromones such as antheridiogen. Three week-old, cordate gametophytes were transferred from multispore cultures grown on nutrient agar to agar containing extracts from a previous generation of gametophytes (treatment) and to fresh nutrient agar (control). Three weeks after transfer, fifty gametophytes were examined from treatment and control plates. Each gametophyte was measured for size (area) and shape (circularity) and scored for number of antheridia and archegonia. Treatment gametophytes were significantly smaller, less circular, had fewer archegonia, and possessed antheridia more often than control gametophytes, a pattern consistent with known antheridiogen effects on gametophytes of transitional morphology and sensitivity. The experiment was repeated using gametophytes that were six weeks old at time of transfer to treatment and control plates. Treatment gametophytes in the second experiment did not differ significantly in size (area) or length from control gametophytes; however treatment gametophytes were more circular and possessed greater widths and length : width ratios, deeper notches, and fewer archegonia. We present a model in which one or more phytochemicals released by cordate gametophytes increase rates of anticlinal division in the apical meristem. The possibilities that the substances involved are phytohormones involved in the development of a notch meristem and cordate morphology in the source gametophyte, and that antheridiogen may be involved, are explored.

Ferns are not as phytochemically diverse as seed plants (Cooper-Driver, 1985). Nevertheless, the ability of many ferns to interact phytochemically with neighboring plants has been well established. The sporophytes of some species, notably *Dennstaedtia punctilobula* and *Thelypteris normalis*, produce allelopaths that suppress germination and growth of gametophytes of the same or other species of ferns (Munther and Fairbrothers, 1980; Raghavan, 1989; Wagner and Long, 1991) or the germination and growth in neighboring seed plants (Horsley, 1977, 1986; Davidonis and Ruddat, 1974; Lyon and Sharpe, 1996).

The most extensively documented phytochemical interactions among gametophytes involve antheridiogen. Antheridiogen is a water-soluble pheromone produced by cordate gametophytes that induces dark germination, precocious maleness, and subsequently retards growth and morphological development in less-developed, acordate neighbors. First observed by Döpp (1950), antheridiogen has been subsequently documented in numerous families of filicalean ferns (Raghavan, 1989; Chiou and Farrar 1997). Along with genetic load, antheridiogen has been suggested as a mechanism promoting relatively

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high rates of cross-fertilization in a group of plants that, given their potentially bisexual gametophytes, appear predisposed to selfing (Soltis and Soltis, 1987; Schneller et al. 1990).

Allelopathic interactions among fern gametophytes have also been reported in which gametophyte development was retarded in crowded conditions relative to less crowded conditions (Smith and Rogan, 1970) or cordate gametophytes in particular retarded spore germination, growth, and survival in acordate neighbors (Bell, 1958; Bell and Klikoff, 1979; Peterson and Fairbrothers, 1980). In these studies, and those regarding antheridiogen, the possibility of phytochemical interactions between cordate gametophytes has remained unexplored. Cordate gametophytes that can perceive the presence of other cordate gametophytes could enhance their own fitness by accelerating their own growth rate, morphological development, or production of archegonia. Such interactions may be particularly relevant to understanding gametophyte ecology and the evolution of phytochemical interactions in ferns.

We report the results of a bioassay for water-soluble phytochemical interactions between cordate gametophytes of *Athyrium filix-femina* var. *asplenoides*, a species with a previously documented antheridiogen system (Schneller, 1979). In isolation, gametophytes of *A. filix-femina* remain asexual until they develop a cordate morphology, when they produce archegonia (Schneller 1979). In multispore populations, acordate gametophytes often become male in response to native antheridiogen and both male and female gametophytes are capable of becoming hermaphrodites following prolonged growth (Schneller, 1979). Thus, *A. filix-femina* possesses a category B pattern of gender expression (Klekowski and Lloyd, 1968).

Materials and Methods

To collect water-soluble pheromones, spores of *A. filix-femina* were collected in the summer of 1996 from Kanawha State Forest in Charleston, WV, and stored in glass vials at room temperature. The spores were then surface sterilized (Dyer 1979) and sown on nutrient agar containing Parker's macronutrients and Thompson's micronutrients (Klekowski, 1969). The resulting gametophtyes were grown for fifteen weeks at a mean temperature of 19.6°C ($\pm$ 0.64) and under a bank of grow lights with a mean light level of 27.0/m^2/sec. ($\pm$ 2.8) and a sixteen hours light : eight hours dark regimen. At the end of fifteen weeks, the gametophytes were discarded and an extract from the agar was obtained by a freeze-thaw process. Suspended matter was removed from the extract by centrifugation and the supernatant was diluted by fifty-percent using Parker-Thompson's nutrients. Fresh agar was then added and the resulting solution was used to make a treatment agar.

In the first experiment, over one hundred three-week old cordate gametophytes (i.e., exhibiting conspicuous apical notches) were transferred to petriplates containing either treatment agar or a control containing basal nutrient agar (Figure 1). No antheridia or archegonia were observed in cordate gametophytes at the time of transfer. Gametophytes were evenly spaced at

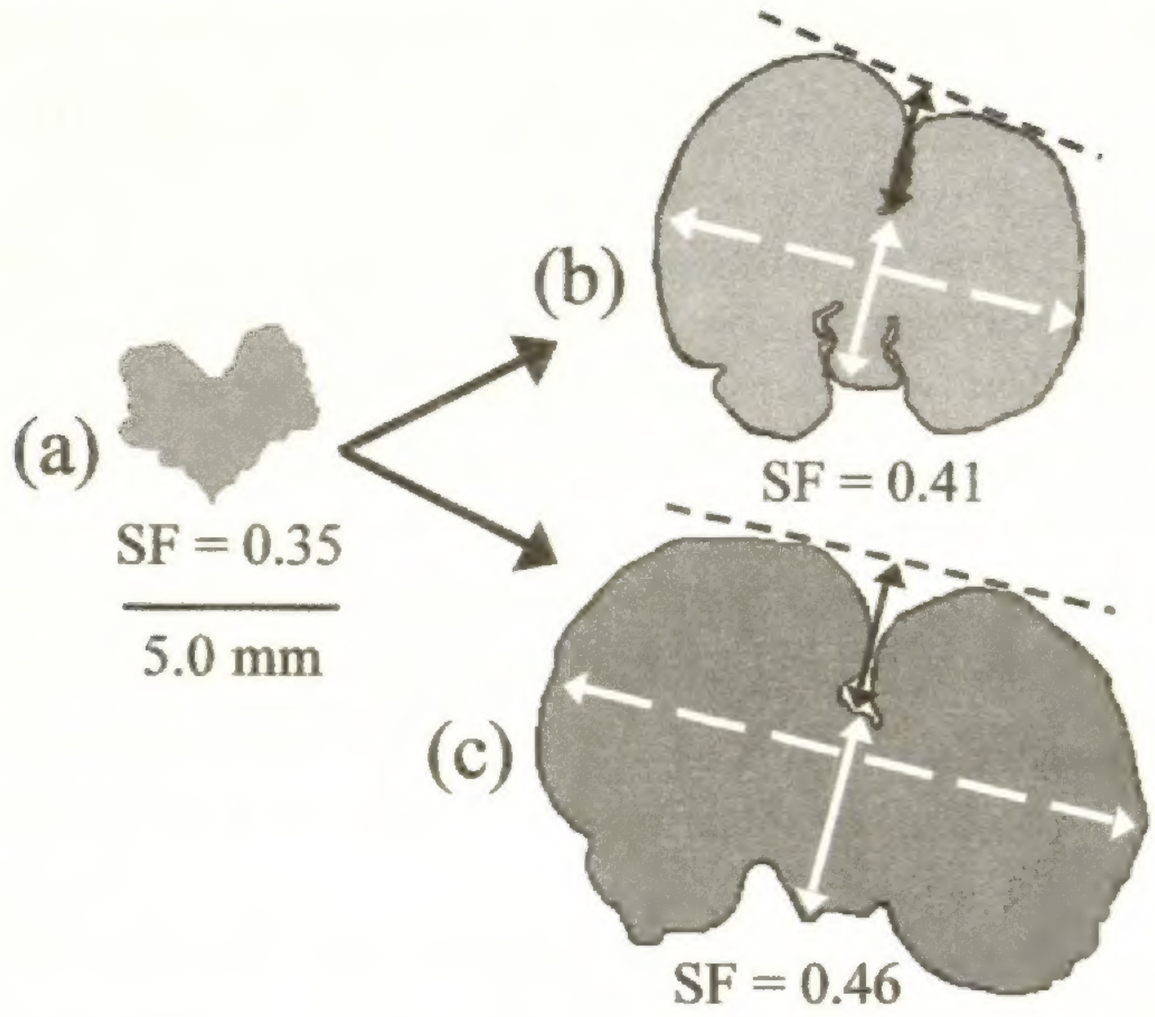

FIG. 1. Gametophyte silhouettes illustrating relative sizes and shapes at beginning and end of second experiment: (a) six-week old gametophyte at time of transfer, (b) largest gametophyte in control population and (c) largest gametophyte in treatment population at end of experiment. Shape factor (SF) is listed immediately below each gametophyte and arrows within in each gametophyte illustrate linear measurements. Gametophyte length was measured from the base of a gametophyte to its notch (solid white arrow) and width was measured as the longest line (dashed white arrow) perpendicular to the length line. Notch depth (solid black arrow) was measured as an extension of the length line from the deepest point in the notch to the line connecting the most distal points of the apical lobes (dashed line).

a density of approximately one gametophyte per square centimeter. Petri-plate lids remained unsealed to facilitate gaseous exchange and reduce ethylene buildup. Twenty-one days after transfer, fifty gametophytes per control and treatment were harvested and mounted on microscope slides using permount. In a second experiment, the entire procedure was repeated using six-week old cordate gametophytes (Figure 1a) at time of transfer and harvesting fifty-five gametophytes per control and treatment. Archegonia in various states of development were observed on all individuals at time of transfer in this experiment. No antheridia were observed.

Each gametophyte was photographed at 8.5× using a digital camera attached to a dissection microscope. Gametophyte photographs were magnified 2× on a computer and size (area in pixels) and shape (circularity) of each gametophyte was measured using Sigmascan Pro 4.0 (Fox and Urich, 1993). Circularity was analyzed using the shape factor function and was calculated using the formula $(4\pi \times \text{Area}) / \text{Perimeter}^2$. Shape factor values range from zero to one, indicating linearity and circularity, respectively. In addition to size and shape, gametophyte length, width and notch depth were also measured in the second experiment using the linear distance function in Sigmascan Pro 4.0. Gametophyte length was measured from the base of the gametophyte to its notch (solid white arrow) and width was measured as the longest line (dashed white arrow) perpendicular to the length line (Figure 1). Notch depth (solid black arrow) was measured as an extension of the length line from the deepest point in the notch

TABLE 1. Equal variance t-tests and Mann-Whitney U rank sum tests of size and shape, and chi-square tests of frequency of antheridia-bearing individuals and archegonia per gametophyte, of three-week old and six-week old *A. felix-femina* gametophytes transferred to basal nutrient agar (control) versus a nutrient agar that supported a previous generation of gametophytes (treatment). Shape factor values approaching zero indicate a linear morphology, whereas those approaching one indicate a circular morphology.

Trait	Control	Treatment	Statistic, P-value
Three-week old gametophytes (at transfer)			
# of Gametophytes	50	50	—
Size (cm^2)*	1.10 ± 0.46 cm^2	0.71 ± 0.36 cm^2	$T = 5.01$, $P < 0.001$
Shape factor	0.28 ± 0.18	0.26 ± 0.18	$U = 1.16$, $P = 0.123$
Antheridial gametophytes/ population	5	19	$X^2 = 10.44$; $P \leq 0.005$
Archegonia/population (per gametophyte)	759 (14.3 ± 9.3)	405 (7.6 ± 7.2)	$X^2 = 109.38$; $P \leq 0.001$
Six-week old gametophytes (at transfer)			
# of Gametophytes	55	55	—
Size (cm^2)*	1.09 ± 0.32 cm^2	1.02 ± 0.23 cm^2	$T = 1.35$, $P = 0.090$
Shape factor	0.40 ± 0.24	0.49 ± 0.22	$U = -2.44$, $P = 0.007$
Length	76.8 ± 12.7	75.4 ± 12.11	$T = 0.51$, $P = 0.30$
Width	152.0 ± 19.2	165.8 ± 31.3	$U = 1980$, $P = 0.006$
Length : width ratio	0.51 ± 0.09	0.47 ± 0.02	$T = 1.98$, $P = 0.03$
Notch depth	31.2 ± 7.6	37.6 ± 10.2	$T = 3.35$, $P < 0.001$
Antheridial gametophytes/ population	0	1	—
Archegonia/population (per gametophyte)	1688 (33.1 ± 13.2)	1461 (28.7 ± 11.5)	$X^2 = 16.38$, $P \leq 0.001$

* Converted from image area in pixels to reflect true size.

to the line connecting the most distal points of the apical lobes (dashed line, Figure 1). Numbers of archegonia and antheridia on each gametophyte were counted directly using a compound microscope in both experiments.

Size and shape data were analyzed using equal variance t-tests, except when data failed to meet assumptions of normality and homoscedasticty, in which case Mann-Whitney U rank sum tests were performed. Data sets were tested for normality using Pearson-D'Agostino omnibus tests and for homoscedasticity using modified-Levene tests. A critical value of $P = 0.05$ was used for all statistical procedures. All statistical analyses were conducted using NCSS 97 (Hintze, 1997).

RESULTS

In the first experiment, individuals from the treatment population were not significantly different in shape, but were significantly smaller, possessed fewer archegonia, and contained more antheridial individuals (both as males and bisexuals), than individuals from the control population (Table 1). Thus, at three-weeks from germination, 38% of cordate gametophytes were still able to respond to antheridiogen by producing antheridia.

In the second experiment, in which six-week old gametophytes were transferred, the treatment population was more circular, possessed greater widths, smaller length : width ratios and deeper notches than the control population (Table 1). Collectively, these observations indicate that lateral growth generated by the apical meristem was greater in the treatment population than in the control population. The treatment population also possessed significantly fewer archegonia (13.3%) than the control population (Table 1). Differences in length and the proportion of males in each population were not significant (Table 1); however, the marginal P-value (0.09) associated with the t-test for difference in size (Table 1) may indicate a weak negative treatment effect. No antheridia were found in the control population and only one gametophyte (1.8%) possessed antheridia in the treatment population.

Discussion

Pheromonal interactions between cordate and acordate gametophtyes, mediated through antheridiogen secretion by the former and uptake by the latter, are known for many filicalean ferns (Näf et al., 1975; Raghavan, 1989). Cordate gametophytes undoubtedly release numerous other substances into their surroundings that may include water-soluble regulators of their own growth and production of gametangia. These substances may influence neighboring cordate gametophytes. Such interactions may provide insight into the phytochemical regulation of gametophyte development and reproductive ecology.

Results from our second experiment demonstrate that cordate gametophytes of *A. filix-femina* produce one or more water-soluble substances that accelerate lateral growth, and subsequent development of a circular profile and deeply recessed notch meristem, and retard production of archegonia in cordate gametophytes of the same species. The ecological relevance of these cordate-cordate interactions remains unclear, however, it is not difficult to envision a fitness advantage to reducing the risk of polyembrony in dense populations with a high likelihood of fertilization success.

Concentrations of the phytochemicals used in this study are unlikely to occur in nature. Although the treatment agar was diluted by fifty percent, it represented the accumulation of fifteen weeks of water-soluble metabolites. In the wild, drainage and biotic and abiotic interactions within soil probably reduced concentrations of these phytochemicals well below those used here. Nevertheless, Greer and McCarthy (1997) observed a peak in cordate males in populations growing on soil at the periphery of the antheridiogen neighborhood (the horizontal range of effect from a source gametophyte) of *Polystichum acrostichoides* (Michx.) Schott. Thus, the substance(s) responsible for the effects observed in this study may reach sufficient levels in nature to induce the responses we observed.

The seemingly contradictory effects of increased circularity without increased size may be the result of one or more water-soluble hormones involved in gametophyte morphogenesis. Morphological development in most filicalean

gametophytes is a function of the planes of division occurring in the meristem (Raghavan 1989). Transition from a one-dimensional filamentous morphology to a two-dimensional spathulate morphology involves the initiation of oblique and anticlinal divisions, as opposed to periclinal divisions, in the single-celled meristem (von Aderkas and Cutter, 1983; Raghavan, 1989). Likewise, transition from a spathulate to a cordate morphology results from an increase in anticlinal divisions in the meristem and its derivatives (von Aderkas and Cutter, 1983; Raghavan, 1989). In cordate gametophytes, anticlinal divisions in the meristem tend to produce small, columnar cells, whereas periclinal and oblique divisions tend to produce larger cells that have a greater impact on overall size (von Aderkas and Cutter, 1983). Thus, the presence of one or more phytochemicals in the treatment may have accelerated anticlinal divisions at the expense of oblique or periclinal divisions, resulting in a deeper notch and wider, more circular profile without a corresponding increase in size. This may also explain the decreased production of archegonia in the treatment in the experiment using six-week old gametophytes, because archegonia are ultimately derived from oblique and periclinal divisions of the meristem.

Although the identity of the substance or substances inducing the effects observed in this study remain unknown, antheridiogen is a candidate. Antheridiogen is a stable, water-soluble compound produced by cordate gametophytes that overrides the light requirement for spore germination, induces precocious maleness in filamentous and spathulate gametophytes, and subsequently retards growth and morphological development in acordate neighbors (Näf et al., 1975; Raghavan, 1989). Structurally, antheridiogen is similar to gibberellin (Näf et al., 1975; Yamauchi et al., 1996; Nester-Hudson et al., 1998), which has similar effects on many seed plants; i.e., it stimulates seed germination and induces maleness in flowers. Recognizing the structural and functional similarity between many known antheridiogens and gibberellin, and the ability of gibberellins to substitute for the antheridiogen of some species of ferns (Näf et al., 1975; Raghavan, 1989), a few authors have speculated about a hormonal role for antheridiogen (Willson, 1981; Voeller and Weinberg, 1969; Schraudolf, 1985; Greer and McCarthy, 1997).

Greer and McCarthy (1997) advanced two hypotheses that may be relevant here, the hormone-pheromone and multiple-signal hypotheses. The hormone-pheromone hypothesis suggests that antheridiogen is a hormone involved in the development and persistence of a cordate morphology in a source gametophyte. In addition to the similarities between antheridiogen and gibberellin listed above, this hypothesis emphasizes the correspondence between the development of a cordate morphology and the production of antheridiogen, and the ability of gibberellin to stimulate cell division in the shoot apex of seed plants. According to the hormone-pheromone hypothesis, response to antheridiogen changes following attainment of a cordate morphology from production of antheridia, reduced growth, and delayed attainment of circularity, to accelerated attainment of circularity and reduced production of archegonia. This model may explain the inhibitory effect of GA_3 on archegonial development in *Lygodium japonicum* (Takeno et al., 1979) and prolonged production of

antheridia and delayed production of archegonia by cordate gametophytes of *Onoclea sensibilis* exposed to antheridiogen of *Pteridium aquilinum* beginning during acordate stages (Näf et al., 1975). It is noteworthy here that genes (TRA and MAN) that control gender expression and are indirectly responsive to antheridiogen in gametophytes of *Ceratopteris richardii* appear to also regulate sporophyte development in this species (Banks 1997).

Alternatively, the multiple-signal hypothesis suggests that cordate gametophytes release two or more water-soluble pheromones. If a compound other than antheridiogen is responsible for accelerated attainment of a circular profile, it has no apparent effect on acordate gametophytes. Under this scenario, receptivity to pheromonal influences on gametophyte development gradually shifts from antheridiogen during acordate phases to one or more other pheromones that affect cordate gametophytes.

In conclusion, cordate gametophytes of *Athyrium filix-femina* produce a water-soluble compound that accelerates the development of a circular profile and retards production of archegonia in well-developed cordate gametophytes. The identity of the substance eliciting these effects remains unknown, however antheridiogen or another gibberellin like substance is a possibility. To answer the many questions raised by this study, similar experiments need to be conducted using isolated antheridiogen and other water-soluble substances released by fern gametophytes.

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Spore Morphology of the Polypodiaceae from Northwestern Argentina

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ABSTRACT.—The spores of the following genera of Polypodiaceae growing in northwest Argentina were analyzed: *Campyloneurum*, *Microgramma*, *Pecluma*, *Phlebodium*, *Pleopeltis* and *Polypodium*. The study involved analyses of herbarium material using light microscopy and scanning electron microscopy. The spores are monolete, 40–90 µm in major equatorial diameter, eliptic to oblong in polar view and plane to concave-convex in equatorial view. The exospore ranges from 2–5 µm thick, is apparently double-layered, with a verrucate or tuberculate surface that is usually perforated. The perispore ranges from 0.3–1 µm thick, is apparently single-layered, attached to the exospore, perforated, and generally smooth or in some cases micro-ornamented. Most of the taxa analyzed have globules on the surface. These are single or associated in masses and irregularly distributed. Characteristics such as size, shape and exospore and perispore sculpture allow us to differentiate among some of the genera as well as recognize species groups. *Microgramma*, *Campyloneurum*, *Pecluma*, *Pleopeltis* and *Polypodium* have verrucate spores whereas those of *Phlebodium* are tuberculate.

This study forms part of a project dealing with the palynological flora of Northwest Argentina. According to de la Sota (1973), this region comprises the provinces of Jujuy, Salta , Tucumán, Catamarca, the eastern part of La Rioja, and southwestern Santiago del Estero (Fig. 1).

The following members of Polypodiaceae grow in this region: *Polypodium argentinum* Maxon, *P. bryopodum* Maxon, *P. chrysolepis* Hook., *P. lasiopus* Klotzsch, *P. loriceum* L., *P. pleopeltidis* Fée, *P. squalidum* Vell., *P. tweedianum* Hook., *Campyloneurum aglaolepis* (Alston) de la Sota, *C. lorentzii* (Hieron.) Ching, *C. major* (Hieron. *ex* Hicken) Lellinger, *C. tucumanense* (Hieron.) Ching, *Microgramma squamulosa* (Kaulf.) de la Sota, *Pecluma filicula* (Kaulf.) M. G. Price, *P. oranense* (de la Sota) de la Sota, *P. venturi* (de la Sota) M.G. Price, *Phlebodium pseudoaureum* (Cav.) Lellinger and *Pleopeltis macrocarpa* (Bory ex Willd.) Kaulf. (de la Sota, 1960, 1977; Ponce,1996). *Polypodium loriceum L.* recently collected by Martínez and de la Sota, (Sota, et al. 1999) in Salta province, was also included in the study. According to Ponce (1996), *Polypodium hirsutissimun* probably grows in the region, however, no material documenting its occurrence was found in the herbaria. According to Tryon and Tryon (1982) and Tryon and Lugardon (1991), American Polypodiaceae are mostly diploid.

The spores of the Polypodiaceae have been described and illustrated with LM by Nayar and Devi (1964), Lloyd (1981) and Pal and Pal (1970), with SEM

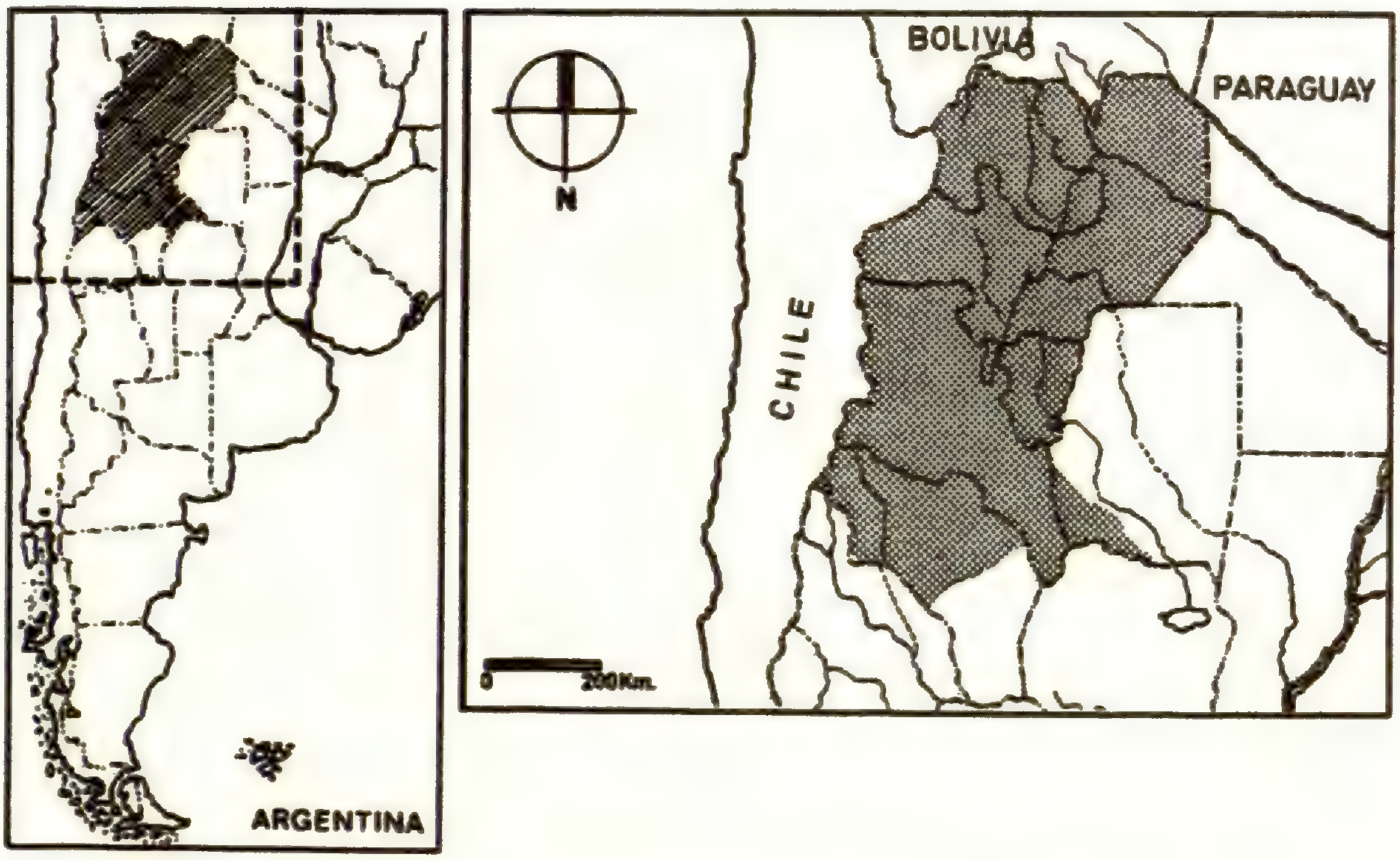

FIG. 1. Study area (Northwest Argentina).

and TEM by Tryon and Lugardon (1991), Tryon and Tryon (1982), van Uffelen (1992, 1993, 2000), van Uffelen and Hennipman (1985) and Hennipman (1990). However among these contributions there are not many references to the spores of species that grow in Argentina.

Tryon and Tryon (1982) differentiated 6 informal groups of *Polypodium* species from tropical America. Those groups were based on a combination of characters such as stem scales, lamina indument, venation, soral arrangement and spores.

The aim of this study is to analyze the spores of the Polypodiaceae that grow in northwestern Argentina in order to add to the existing information about these taxa and to assess the systemic value of palynological data.

MATERIALS AND METHODS

Spores were obtained from herbarium (BA, LP, LIL and SI) specimens and were studied using light (LM) and scanning electron microscopy (SEM). For LM the spores were treated with hot 3% sodium carbonate for 2 minutes and acetolyzed according to the method of Erdtman (1960). For SEM, the material was treated with hot 3% sodium carbonate, washed, dehydrated, suspended in 96% ethanol and then transferred to acetate plates. After dryng they were coated with gold. Wall fractures obtained using ultrasound for 10 minutes were also used in order to study the sporoderm structure. All the observations were made with Olympus BH2 and BHB light microscopes and a JEOL JSMT- 100 scanning electron microscope at the Museo de Ciencias Naturales de La Plata.

The terms proposed by Hennipman (1990), Tryon & Lugardon (1991), van Uffelen & Hennipman (1985) and van Uffelen (1993) were used for spore descriptions.

Spores were characterized for: color of acetolyzed material, shape, diameters, laesurae, sporoderm thickness, and ornamentation, structure and stratification of the wall layers. In some species of *Campyloneurum*, the number of spores produced per sporangium was considered in order to understand spore irregularities related to size and morphology.

The letters MP, associated with the list of specimens investigated (Table 1) indicate the reference number of each palynological sample as filed in the Laboratorio de Palinología, Facultad de Ciencias Naturales y Museo de La Plata.

Results

Campyloneurum (Table 2; Fig. 2, A–L)

This genus is represented by four species in the study area: *C. aglaolepis* (Alston) de la Sota, *C. lorentzii* (Hieron.) Ching, *C. major* (Hieron. ex Hicken) Lellinger and *C. tucumanense* (Hieron.) Ching. All are epiphytic and characterized by an entire lamina, anastomosing veins and round sori borne in a marginal or submarginal position.

Campyloneurum tucumanense is the largest species of the genus in the northwestern of Argentina. The lamina is soft, membranaceous and has the most complex and evident venation of the *Campyloneurum* studied. Ponce (1996) considered this species to be endemic to the region of study although it has also been reported for Bolivia by Lellinger (1988).

The spores are ellipsoidal or oblong in polar view (Fig. 2 A, D and J) and plane to concave-convex in equatorial view (Fig. 2 B, F, G and K) . In *Campyloneurum aglaolepis*, *C. lorentzii*, and *C. major*, equatorial diameters range between 70 and 80 μm and polar diameters between 40 and 60 μm. In *Campyloneurum tucumanense* the equatorial diameter ranges between 85 and 94 μm and 46–60 μm in polar diameter.

The exospore is the thickest wall layer, ranging between 1.5 and 3 μm in all species except in *Campyloneurum tucumanense* in which it reaches 4 μm. Spores are verrucate with a dense compact wall (Fig. 2 I). With LM it is apparently double-layered in section with a compact structure. The inner layer (ie) is thiner than the outer (ie: oe ratio 1:2–1:3). The outer layer (oe) forms the elements of the sculpturing (verrucae). The verrucae have a circular or polyhedral outline and diminish in size toward the proximal face. In *C. aglolepis* (Fig. 2 A–C) the verrucae are obscure. The verrucae of *C. aglolepis* (Fig. 2 A–C) and *C. lorentzzi* (Fig. 2 D–F) are larger than in *C. major* (Fig. 2 G–I) and *C. tucumanense* (Fig. 2 J–L).

The perispore is 0.4–1 μm thick, smooth and perforated. With LM it is aparently single-layered in section and follows the verruca contours (Fig. 2 C, E and H). Most of the species analyzed have irregularly distributed globules on

TABLE 1. Specimens studied.

Taxon	Voucher specimens
Polypodium L.	
P. argentinum Maxon	Jujuy: Dpto.Yala, *Eskuche 119* (LP), MP 3853 Salta: Dpto. Santa Victoria, *Hurrel 51* (LP), MP 3852 Tucumán: Dpto. Tafí, *Maruñak, Olivia & Puezo 345* (LP), MP 3847
P. bryopodum Maxon	Jujuy: Dpto. Tumbaya, Volcán, *Cabrera, Torres, Tur & Kiesling 18353* (LP), MP 3846 Tucumán: Dpto. Chicligasta, Estancia Santa Rosa, *Venturi 4802* (LP), MP 3851
P. chrysolepis Hook	Salta: Dpto. Orán, cerro La cueva, (SI, *1321*), MP 3913. Tucumán: Dpto Tafi, Río Potrero, *Bruchter s/n* (LP), MP 3845 Tucumán: Dpto Tafi, La Ventanita, *Castillo 35* (SI), MP 3914
P. lasiopus Klotzsch	Jujuy: Dpto. Capital, Laguna de Yala, *Palací et al. 893* (SI), MP 3884 Salta: Dpto. Guachipas, Estancia Pampa Grande, *Hawkes et al. 3976* (LP), MP 3887
P. loriceum L.	Salta: Dpto Santa Victoria, Los Toldos, *Martínez 641 et al.* (LP), MP 3886 Salta: Dpto Santa Victoria, Los Toldos, *Martínez et al. 595* (LP), MP 3957
P. pleopeltidis Fée	Jujuy: Dpto. Ledesma, *Cabrera, Kiesling & Zardini 24008* (LP), MP 3849 Jujuy: Dpto. Ledesma, Abra de las Cañas, *de la Sota 4428* (LP), MP 3917.
P. squalidum Vell.	Jujuy: Dpto. Santa Bárbara, *Zuloaga & Deginami 327* (LP), MP 3882 Jujuy: Dpto Ledesma, Calilegua, Arroyo del medio, *Cabrera et al 30363* (LP), MP 3956 Salta: Dpto Oran, Parque Nac. El Rey, Arroyo La Sala, *Brown 90* (LP), MP 3844
P. tweedianum Hook.	Jujuy: Dpto. Capital, Lozano, *Krapovickas & Schinini 35825* (LP), MP 3881 Salta: Dpto. Orán, Aguas Blancas, *Palací 104* (LP), MP 3885 Tucumán: Dpto. Monteros, Quebrada Pueblo Viejo, *de la Sota 4066* (LP), MP 3848
Campyloneurum *C. Presl*	
C. aglaolepis (Alston) de la Sota	Salta: Dpto. Capital, San Lorenzo, *Cabrera 3061* (LP), MP 3888 Salta: Dpto. Capital, Quebrada San Lorenzo, *Cabrera 9118* (LP), MP 3916
C. lorentzii (Hieron.) Ching	Salta: Dpto. Capital, Quebrada San Lorenzo, *Palací 160* (LP), MP 3889 Salta: Dpto. Capital, Parque Nacional El Rey, *Brown 983-2* (LP), MP 3954 Tucumán: Burruyacú, Los Pinos, *Borsini s/n* (LP), MP 3952 Jujuy: Dpto. Capital, Quebrada Yala, *Cabrera y Kiesling 25227* (LP), MP 3953
C. major (Hieron. *ex* Hicken) Lellinger	Jujuy: Dpto. Ledesma, Mesada de las Colmenas, *de la Sota 4483* (LP), MP 3941

TABLE 1. Continued.

Taxon	Voucher specimens
C. tucumanense (Hieron.) Ching	Jujuy: Dpto. Ledesma, 10 a 20 km de Libertador Gral. San Martín, *Krapovickas, Schinini & C. Quarín 26641* (LP), MP 3900 Salta: Dpto. Sta. Victoria, Baritú, *Marmol, Legname & Cuezzo 8762* (LP) MP 3902 Tucumán: Dpto. Tafí, Quebrada de Tafí, *Venturi 871* (LP), MP 3901
Pecluma M.G. Price	
P. filicula (Kaulf.) M.G. Price	Jujuy: Dpto. Valle Grande, Mesada de las Colmenas, *Fabris 3425* (LP), MP 3912 Salta: Dpto. Orán, Aguas Blancas, Quebrada El Nogal, *Palací 92* (LP), MP 3911
P. oranense (de la Sota) de la Sota	Jujuy: Dpto. Capital, Cerro Labrado, *de la Sota 4310* (LP), MP 3909 Salta: Dpto. Santa Victoria, *Mármol, Cuezzzo (h) & Cuezzo 9209c* (LP), MP 3910
P. venturi (de la Sota) M.G. Price	Salta: Dpto. Capital, Quebrada San Lorenzo, *Palací 166* (LP), MP 3890 Salta: Dpto. Santa Victoria, Los Toldos, Quebrada El Astillero, *Palací 499* (LP), MP 3907 Tucumán: Dpto. Monteros, Quebrada Pueblo, *de la Sota 4059* (LP), MP 3908
Microgramma C. Presl	
M. squamulosa (Kaulf.) de la Sota	Jujuy: Dpto. Capital, La Cuesta, *Cabrera et al. 18856* (LP), MP 3904 Salta: Ruta Nac. 9, Pampa Grande, *Calandra s/n* (LP), MP 3903 Tucumán: Dpto. Monteros, Quebrada de los Sosa, Casa de Piedras, *Krapovickas & Cristóbal 20456* (LP), MP 3905
Phlebodium *(R. Brown) J. Smith*	
P. pseudoaureum (Cav.) Lellinger	Jujuy: Dpto. Capital, *Cabrera 8178* (LP), MP 3906 Salta: Dpto. Rosario de Lerma, *Venturi 8227* (LP), MP 3850 Salta: Dpto. Orán, Aguas Blancas, *Palací 96* (LP), MP 3918
Pleopeltis *Humb. & Bonpl. ex Willd.*	
P. macrocarpa (Bory ex Willd.) Kaulf.	Salta: Dpto. Santa Victoria, Los Toldos, *Martínez et al. 653* (LP), MP 3891 Salta: Dpto. Santa Victoria, camino a Los Toldos, *Martínez et al. 644* (LP), MP 3915

the spore surface (Fig. 2 C, D, G, J and K). In some samples (*de la Sota 4483*, LP; Fig. 2 H) small perforations were observed across the perispore surface.

The number of spores produced per sporangium was estimated in several specimens in order to check for the possiblity of apogamy in *Campyloneurum tucumanense*. In *Krapovickas et al. 26641* (LP) and in *Venturi 871* (LP), 64 spores per sporangium were estimated and, apart from mature spores, hyaline

TABLE 2. Spore morphological data of the Polypodiaceae from Northwestern Argentina (sizes in µm, mean value in parentheses).

Taxon	Major equatorial diameter	Minor equatorial diameter	Polar diameter	Laesura length	Exospore	Perispore	Exospore ornamentation
Polypodium argentinum	76.6 (86.2) 95.6	54 (61.3) 68.5	46 (54.8) 64	46 (52.7) 58.6	2.5 (3.3) 4.3	0.4 (0.8) 1	Verrucate, with globules
P. bryopodum	61.9 (75.6) 87.1	54.7 (61.9) 72	34.9 (45.2) 61.5	44.8 (55.4) 55.2	3.1 (3.9) 4.9	0.4 (0.7) 0.8	Verrucate, with globules
P. chrysolepis	71.4 (78.4) 82.1	48.1 (54.6) 61.8	38.9 (48.1) 56.2	34.4 (40.8) 46.3	1.8 (3.0) 3.4	0.4 (0.6) 0.6	Verrucate, with globules
P. lasiopus	74 (83) 94.8	43.3 (57.2) 64.1	39.9 (52.1) 59.8	30 (38) 41.5	2.1 (3.3) 4.1	0.4 (0.7) 0.9	Verrucate, with large verrucae and ridges
P. loriceum	51.6 (63.5) 74.5	37.2 (40.5) 45	30.4 (36.7) 47.8	41.5 (45.2) 49.8	2.9 (3.4) 4.2	0.4 (0.6) 0.8	Verrucate, with large verrucae and ridges
P. pleopeltidis	58.5 (66.1) 75.7	37.2 (42.1) 49.5	32.5 (42.6) 46	31.6 (34.5) 38.6	2.1 (2.9) 3.9	0.8 (1.1) 1.2	Verrucate, with large verrucae
P. squalidum	47.8 (57.2) 64.7	35.1 (40.4) 46.7	33.3 (39.6) 45.2	22.5 (26.9) 31.2	1.8 (2.15) 2.5	0.57 (0.7) 1	Verrucate, with globules
P. tweedianum	59.5 (69.6) 80.7	41.8 (47.4) 51	30.5 (37.4) 44.4	44.8 (50.1) 58	1.7 (2.1) 2.5	0.3 (0.4) 0.6	Verrucate, with low verrucae, and globules
Campyloneurum aglolepis	67.6 (73.8) 83	47.7 (54.9) 64.7	47.3 (51.1) 55.6	29.5 (34.6) 39.4	2.1 (2.4) 2.9	0.7 (0.8) 0.9	Verrucate, with low verrucae, and globules
C. lorentzii	64.9 (71.5) 79.1	54.8 (59.2) 63.9	41.8 (48.9) 55.9	26.0 (30.9) 39.1	2.2 (2.9) 3.3	0.5 (0.7) 0.8	Verrucate, with globules
C. major	71.4 (71.8) 72.2	39.0 (45.2) 51.5	37.3 (42.5) 45.6	44.8 (45.2) 45.6	1.5 (1.6) 1.7	0.4 (0.5) 0.8	Verrucate, with few globules
C. tucumanense	85.1 (91.0) 93.8	57.3 (63.6) 71.4	46.5 (54.3) 59.3	45.6 (53.9) 61.4	3.3 (3.7) 4.1	0.8 (0.9) 1.3	Verrucate, with few globules
Pecluma filicula	45.1 (51.9) 58	31.7 (37.7) 40.2	26.9 (32.5) 39.1	21.2 (27.1) 30.7	1.4 (1.7) 2.1	0.6 (0.8) 0.95	Verrucate, with few globules
P. oranense	44 (49.6) 54	27.5 (28.5) 33	26 (27.9) 30.5	20.5 (25.65) 30	2 (2.6) 3.4	0.5 (0.8) 1	Verrucate, with large verrucae in two levels and ridges, with globules
P. venturi	43.6 (48.7) 54	28.1 (33.1) 36.4	26.5 (30.3) 35.9	21.6 (26.31) 28	1.8 (2.14) 2.3	0.45 (0.6) 0.8	Verrucate, with large verrucae in two levels and ridges, with globules
Microgramma squamulosa	70.5 (75.9) 80.9	52.9 (55.8) 60.5	42.1 (46.6) 52.1	35.6 (38.1) 43.6	3.1 (4.1) 4.9	0.4 (0.5) 0.7	Verrucate, with few globules
Phlebodium pseudoaureum	35.6 (38.2) 41.3	21.8 (23.8) 26.7	21.1 (23.5) 25.9	19.4 (20.8) 25.1	3.2 (3.6) 4.1	0.9 (1.0) 1.2	Tuberculate, with few globules
Pleopeltis macrocarpa	74.7 (81.6) 86.3	45.6 (52.1) 64.3	77.6 (80.1) 81.7	41.5 (45.4) 50.6	1.7 (2.0) 2.3	0.9 (1.2) 1.5	Verrucate, with few globules

and small immature spores were also observed. In *Legname* & *Cuezzo* 8762 (LP) 32 spores per sporangium were estimated.

Pecluma (Table 2; Fig. 3 A–K)

This genus is represented by three species in the northwest of Argentina: *P. filicula* (Kaulf.) M. G. Price, *P. oranense* (de la Sota) de la Sota and *P. venturii* (de la Sota) M. G. Prince. *Pecluma oranense* is endemic to Salta and Jujuy, growing as an epiphyte in the basal forest and in *Podocarpus parlatorei* dominated forest. This genus is characterized by pinnatifid, pubescent lamina, sori borne at the tip of a vein and nonclathrate basifixied rhizome scales. *Pecluma filicula* is the smallest of the species studied. It reaches ca. 20 cm in length and has a pubescent and scaly rachis, whearas *P. oranenese* and *P. venturi* have longer lamina and have a pubescent, but never scaly rachis.

The spores are monolete and yellowish in *Pecluma oranense* and *P. venturi* and light-brown in *P. filicula*. They are ellipsoidal to oblong in polar view (Fig. 3 A, E, G and H), and concave and convex distally in equatorial view (Fig. 3 B, D and I). They are 44–58 µm in major equatorial diameter and 26–40 µm in polar diameter.

The exospore is 1–2 µm thick in *Pecluma filicula* and 2–3.4 µm thick in *P. oranense* and *P. venturi* . With LM it is apparently double-layered in section and with a verrucate surface. The inner layer (ie) is bright yellow and the outer one (oe) light yellow. In *P. filicula* the ie: oe ratio is 1:1 to 1:2, while in *P. oranense* and *P. venturi* the ie: io is 1:4–1:5.

The exospore of *Pecluma filicula* has heterometric, low verrucae arranged on one level (Fig. 3 A–C). When observed with LM in equatorial view, exospore thickness increases toward the proximal face. The perispore is 0.6–0.9 µm thick, verrucate and perforate. The perforations are located between verrucae (Fig. 3 C). The perispore is uniformly adhered to the exospore.

In *Pecluma oranense* (Fig. 3 D–F) and *P. venturi* (Fig. 3 G–K) the verrucae are arranged in two levels. The upper level has spheres and large verrucae, which are sometimes laterally fused. The lower level has heterometric verrucae, which are sometimes fused but are smaller than the upper ones. In both species verruca size diminishes toward the proximal pole. When observed with LM in equatorial view, exospore thickness diminishes toward the proximal pole.

As seen with LM the perispore is 0.4 to 1 µm thick, apparently single-stratified and adhered to the exospore. It has a micro-verrucate and baculate surface (more evident in *Pecluma oranense* and *P. venturi*;Fig. 3 F, J and K).

The globules in *Pecluma venturi* (Fig. 3 J) showed a micro-verrucate surface like that of the exospore.

Microgramma (Table 2; Fig. 4 A–D)

Only one species, *M. squamulosa* (Kaulf.) de la Sota, is reported for Northwest Argentina. A hybrid, *Microgramma x mortoniana*, was reported for

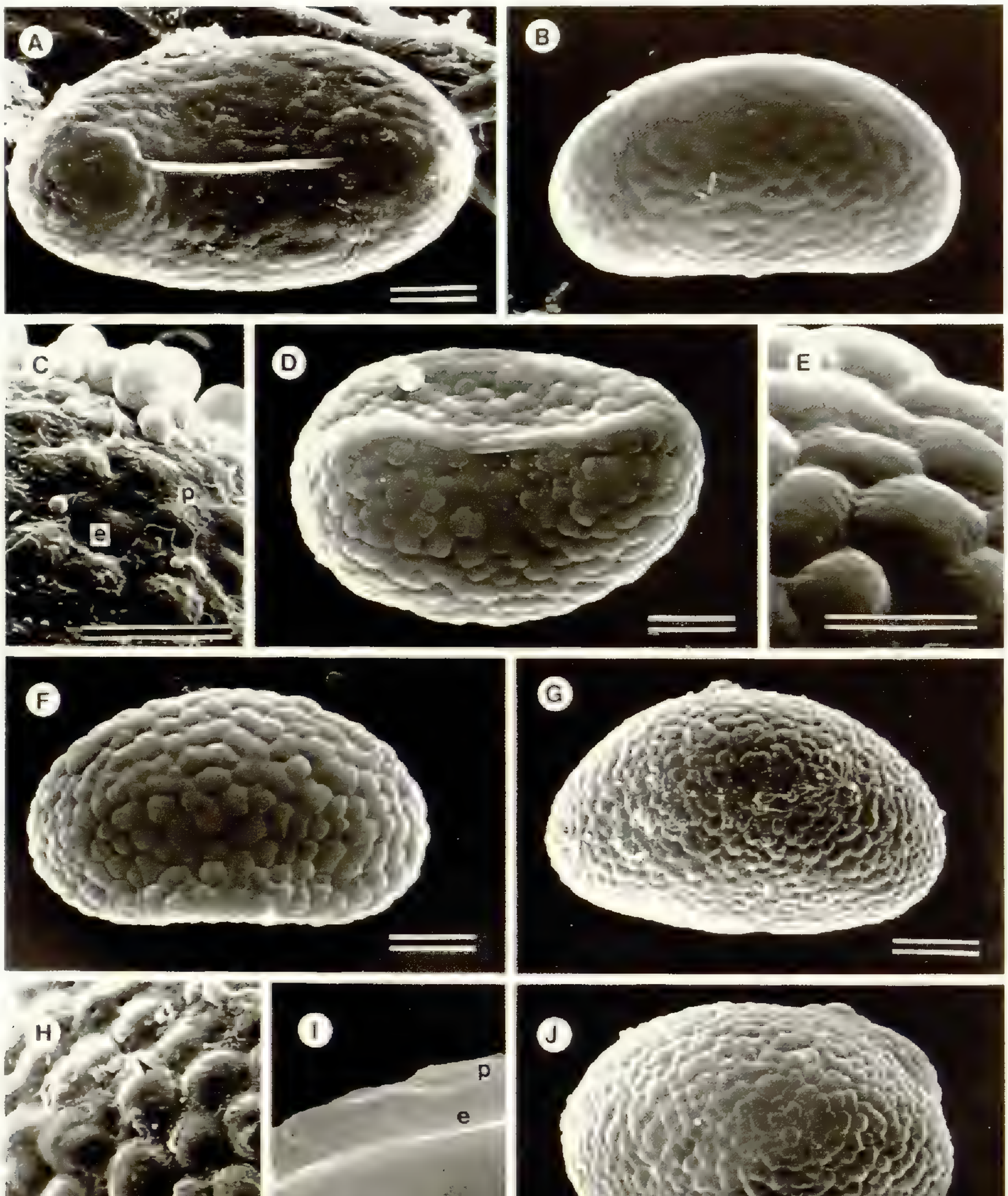

FIG. 2. SEM micrographs of *Campyloneurum* spores.
A–C, *Campyloneurum aglolepis* (*Cabrera 3061*). A. Proximal view. B. Equatorial view. C. Detail of the surface, showing single or grouped globules on the abraded perispore. D–F, *Campyloneurum lorentzii* (*Kiesling 25227*). D. Proximal view, isolated globules are present on the surface. E. Distal surface in detail, the sculpture is slightly verrucate. F. Equatorial view, the verrucae are polyedric

Salta by de la Sota, *et al.* (1999). The hybrid was not included in this study because the specimen was sterile.

The spores of *Microgramma squamulosa* are monolete, yellowish to light brown, ellipsoidal or oblong in polar view (Figs. 4 A, B) and plane/concave-convex in equatorial view, 70–80 μm in major equatorial diameter and 42–52 μm in polar diameter.

The exospore is 3–5 μm thick and apparently double-layered in section when observed with LM. The inner layer (ie) is bright yellow and the outer (oe) light brown. The ie: oe ratio varies from 1:4 to 1:6. The exospore sculpture is verrucate. The verrucae have a micro-verrucate surface (Fig. 4 C–D). The verrucae are single, or fused to form ridges (Fig. 4 A–D) which diminish in size toward the proximal face (Fig. 4 A–B).

The perispore is 0.4–0.7 μm thick, apparently single-layered in section with the LM closely adhering to the exospore and smooth or micro-verrucate with perforations (Fig. 4 C–D). Occasionally, globules are observed on its surface (Fig. 4 C–D).

In spores of *Krapovickas & C.L. Cristobal* 20456 (LP) granular material was observed on the surface (Fig. 4 C).

Phlebodium (Table 2; Fig. 4 E–I)

One species of this genus, *P. pseudoaureum* (Cav.) Lellinger, is present in the northwest of Argentina. It grows as a deciduous epiphyte in the basal forest. The laminae are large, pinnatifid and glabrous with anastomosing venation.

The spores are monolete, yellowish, oblong to ellipsoidal in polar view (Fig. 4 E and G) and plane to concave and convex distally in equatorial view (Fig. 4 F). Their diemnsions are 35–42 μm in major equatorial diameter and 21–26 μm in polar diameter.

The exospore is 3–4 μm thick, at LM apparently double-layered in section, the inner layer (ie) being lighter than the outer layer (oe). The ratio ie: io is 1:3 to 1:5. It is compact as seen in fractures observed with SEM (Fig. 4 I). The exospore is tuberculate (Fig. 4 E–H) and the tubercles are single or fused, with a blunt or truncate apex. They seem to be formed by coalescent roads (Fig. 4 F–H).

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in shape and heterometric, the size of the verrucae diminish toward the proximal pole at both sides of the lesura. G–I, *Campyloneurum major* (*de la Sota 4483*). G. Equatorial view, the verrucae are round, small, and densely packed. H. Distal surface in detail. Irregularly distributed perforations (arrow head) are present and some verrucae are fused. I. Fracture through the sporoderm. J–L, *Campyloneurum tucumanense*. (J and L: *Schinini & C. Quarín 26641*; K: *Legname & Cuezzo 8762*). J. Distal view, globules are occasionally present. K. Equatorial view. Verruca size diminishes toward the proximal pole which is at the bottom. L Detail of the distal surface, the verrucae are heteromorphic and heterometric and some of them fused. Their surfaces are slightly verrucate. Scale bars: A, B, D, F, G, J and K 10 μm; C, E, H and L: 5 μm; I: 2 μm.; e: exospore, p: perispore.

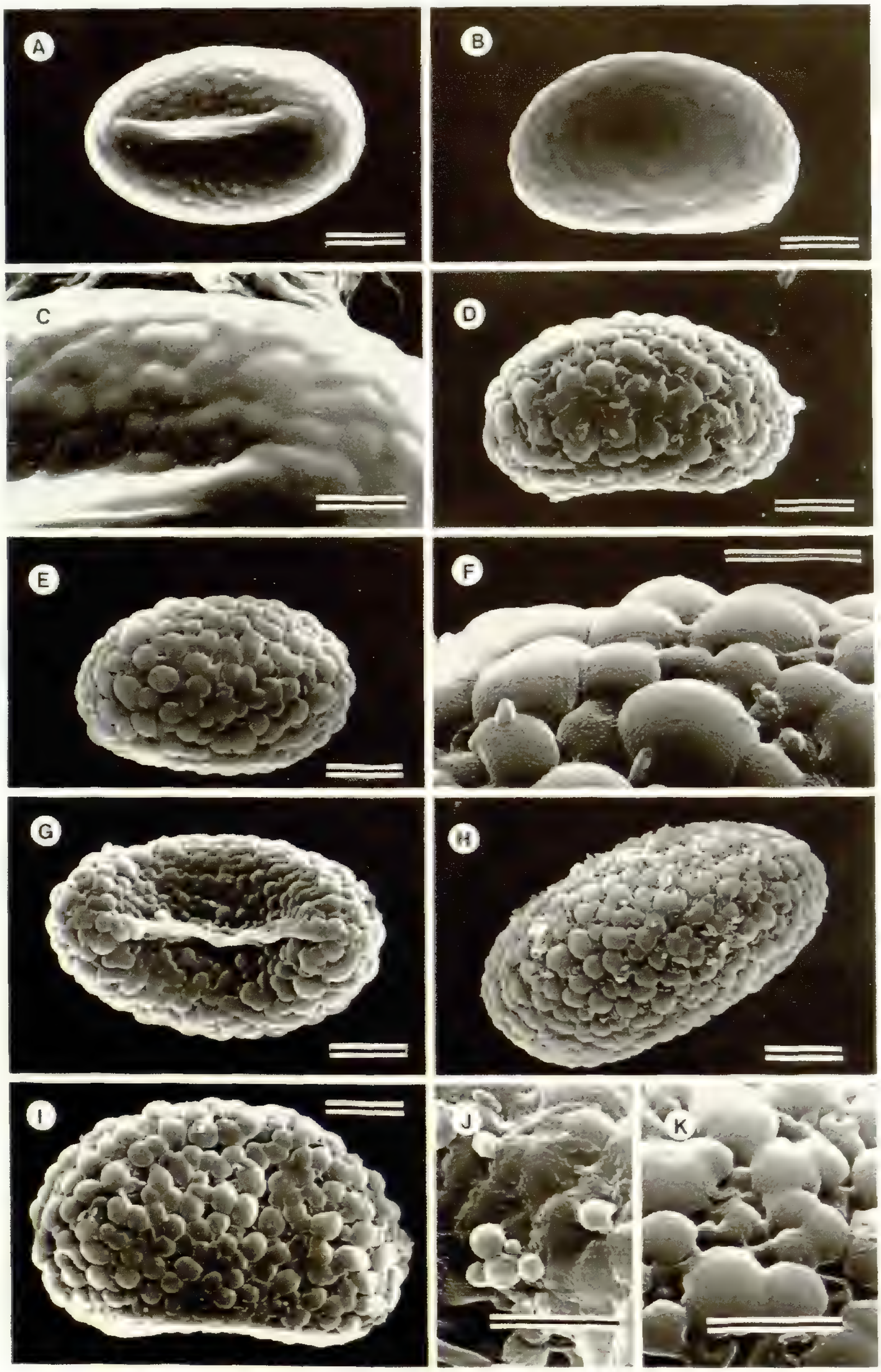

FIG. 3. SEM micrographs of *Pecluma* spores.
A–C, *Pecluma filicula* (*Palací 92*), A Proximal view. Verruca size diminishes toward the proximal pole. The laesura ridge is thick. B. Equatorial view. C. Detail of the proximal surface; verruca surface is smooth and perforations are present between verrucae at the sides of the laesura (arrowhead). D–F, *Pecluma oranense* (*de la Sota 4310*) D. Equatorial view, showing verruca

The perispore is up to 1 μm thick, with LM it is apparently single-layered in section, smooth with few perforations (Fig. 4 H) and adherant to the exospore.

Pleopeltis (Table 2; Fig. 4 J–M)

One species, *P. macrocarpa* (Bory ex Willd.) Kaulf., is reported (Ponce, 1996) for the northwest of Argentina. It grows as an epiphyte or is epipetric in the basal forest between 1000 and 2000 a. s. l. It is characterized by a scarcely pubescent lamina, ellipsoidal sori, and peltate scaly paraphyses.

The spores are monolete, light brown, ellipsoidal to sub-spheroidal in polar view (Fig. 4 J) and plane/concave-convex in equatorial view (Fig. 4 K), 75–86 μm in equatorial diameter and 46–64 μm in polar diameter.

The exospore is 1.7–2.3 μm thick and verrucate. The verrucae are very low, isolated, and have a micro-verrucate surface (Fig. 4 J–L). With LM it is apparently double-layered in section, the inner layer (ie) being brighter than the outer layer (oe). The ie: oe ratio is 1:3–1:4. The exospore is apparently compact, as seen in fractures with SEM (Fig. 4 M).

The perispore is 1 μm thick, and apparently single-stratified with LM and adhered to the exospore. Its surface is smooth or micro-verrucate and perforated (Fig. 4 L). Globules were observed on the surface (Fig. 4 K). In some specimens (*Martínez et al. 644*, LP), there were abundant perforations distributed on the whole surface of the perispore (Fig. 4 L).

Our material of *Pleopeltis macrocarpa* has spores with characteristics similar to those described by Tryon & Tryon (1982) based on material from Peru. These authors illustrated the spores of *P. macrocarpa* with SEM and described the surface as verrucate, with globules and ca. 70 μm.

Polypodium (Table 2; Figs. 5, 6)

Eight species of *Polypodium* grow in the northwest of Argentina. They are *P. argentinum* Maxon, *P. bryopodum* Maxon, *P. chrysolepis* Hook., *P. lasiopus* Klotzsch, *P. loriceum* L., *P. pleopeltidis* Fée, *P. squalidum* Vell, and *P. tweedianum* Hook. These species are epiphytic or rupestral, rarely terrestrial, and are characterized by a pinnatifid lamina, with scales or glandular hairs and with sori borne at the tip of a vein.

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diameter diminishes toward the proximal pole. E. Distal view, showing verrucae at different levels; some of the verrucae are heterometric and fused. F. Magnification of the distal surface of the spore in picture 4; verruca surfaces are micro-verrucate and the verrucae are laterally fused. G–K, *Pecluma venturii* (de *la Sota 4059*) G. Proximal view, the verrucae are smaller here than in the rest of the spore surface and the laesura is membranaceous and high. H. Distal view showing verrucae at different levels with some of them fused. I. Equatorial view, the verrucae are so densely packed that most of them have a polygonal outline, and some are fused. Verrucae diameter diminishes toward the proximal pole. J and K. Magnifications of the surface, showing micro-verrucation of surface. In J, isolated and grouped globules are present on their surfaces; note the similarity between globule and verruca surface. Scale bars: A, B, D, E, G, H, I: 10 μm; C, F, K and J: 5 μm.

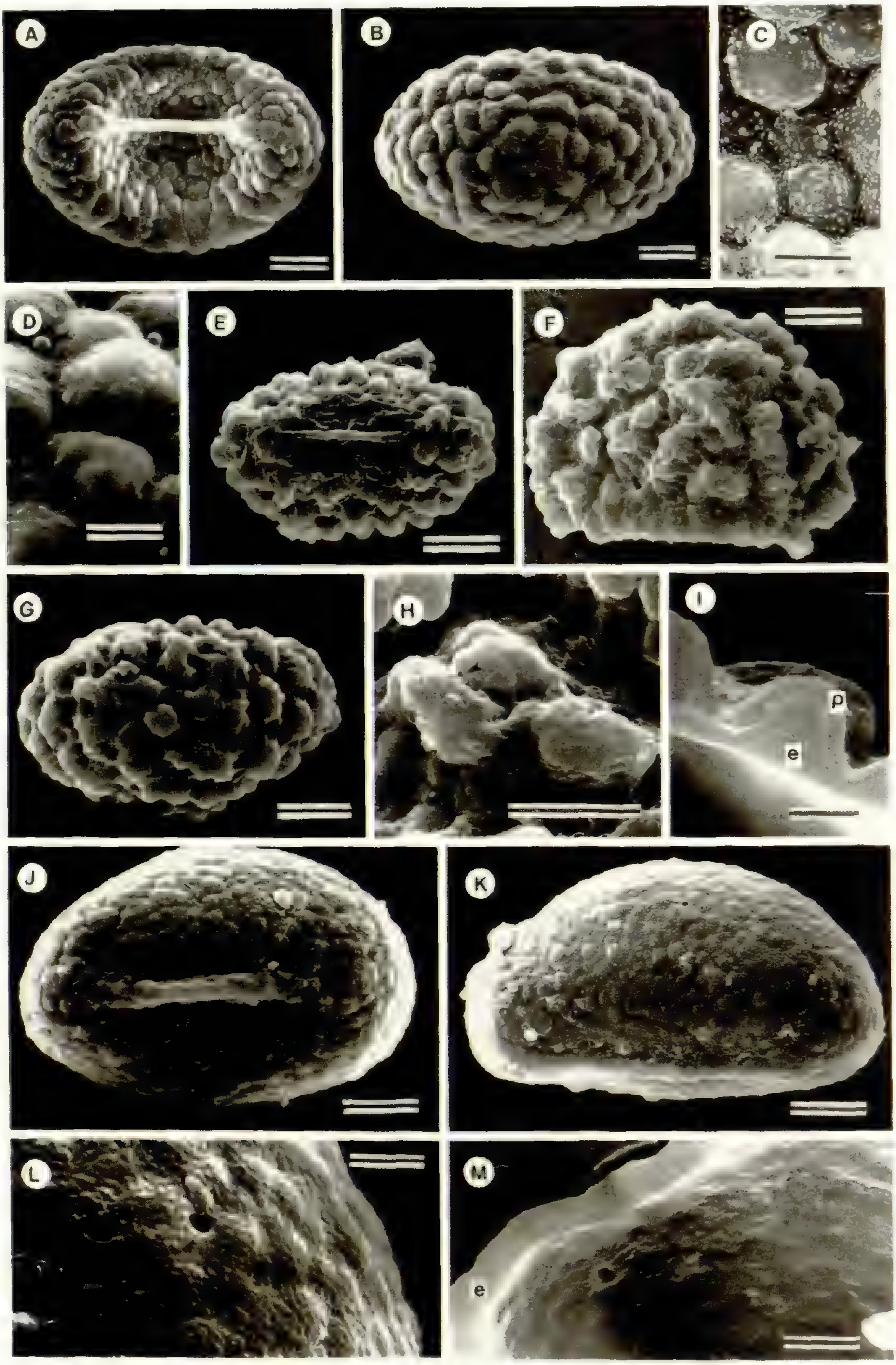

FIG. 4. SEM micrographs of *Microgramma, Phlebodium* and *Pleopeltis* spores.
A–D, *Microgramma squamulosa* (*Krapovickas & Cristobal 20456*) A. Proximal view, the verrucae are smaller on both sides of the laesura. B. Distal view, the verrucae are both irregularly-shaped and irregularly-sized. Some of them are fused, forming ridges. C. Detail of the proximal surface of the spore in A with polygonal verrucae and micro-verrucate surface. Small globules and granular

The spores are monolete, light brown to yellowish, ellipsoidal to oblong in polar view (Fig. 5 A, D, G, and L; Fig. 6 B, C and F) and plane-convex to plane-hemispherical in equatorial view (Fig. 5 B, E, H, K and N; Fig. 6 A, E, H and I), 52–98 μm in equatorial diameter and 29–65 μm in polar diameter.

The exospore is generally 2–4 μm thick, but range to 8 μm thick *in Polypodium lasiopus*, and verrucate. As seen with LM it is apparently double-layered in section and in equatorial view it increases in thickness toward the proximal face. Variation in size and degree of verruca fusion were observed in different specimens.

In all the species the perispore is up to 1 μm thick. With LM it is apparently single-layered in section and smooth, rugulate or micro-verrucate according to the species (Fig. 5 C, F, I, J, M and O; Fig. 6 D, G and J).

There are perforations on the exospore and perispore surfaces in all the analized species, located on and between verrucae (Fig. 5 C and F; Fig. 6 D and J)

Sporopollenin globules either single or associated in masses are adherant to the perispore in most of the species analized. They differ in size, number and distribution. These globules show a structure similar to that of the sporoderm (Fig. 5 B–E, G, H, J–K; Fig. 6 A–C, E–J).

In *Polypodium argentinum* the verrucae are uniform in shape and size, and isoleted or grouped globules are adherant to the perispore (Fig. 5 A–C). The spores of *Polypodium chrysolepis* (Fig. 5 G–J) have low verrucae. The verrucae in *Polypodium lasiopus* (Fig. 5 K–M) and *P. loriceum* (Fig. 5 N–O) are circular to polyhedral and fused, to form radial ridges across the proximal face. The spores of *P. squalidum* are the smallest within the Polypodiacea studied here, the verrucae are uniform in size and shape, and globules are adherant to the perforated perispore (Fig. 6 E–G). The spores of *Polypodium tweedianum* (Fig. 6 H–J) have verrucae that are perforated, polyhedral and variable in size.

According to observations with LM, the globules of *Polypodium bryopodum* (Fig. 5 D–F) and *P. pleopeltidis* (Fig. 6 A–D) show a central zone much dense

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material are abundant on the whole surface. D. Detail of the surface of the spore in B showing micro-verrucate verruca surface and scattered globules (arrow head) of different sizes are present on and between the larger verrucae. E–I, *Phlebodium pseudoareum* (*Cabrera 8178*). E. Proximal view, the elements of the sculpture are low on either side of the laesura; the sculpture is tuberculate. F. Equatorial view, showing fusion of several basal ridges (arrow) to form tubercles. G. Distal view; continuity between basal ridges of continuous tubercules is evident (arrow heads). H. Detail of the equatorial surface with several large, grouped tubercles. Several ridges form the bases of the grouped elements and the spaces between tubercles are deep; perforations indicated by arrow head. I. Fracture across the sporoderm that exposing the juncture between exospore and perispore. J–M, *Pleopeltis macrocarpa* (*Martínez et al. 644*). J. Proximal view. The laesura is a short and the sculpture is verrucate; the verrucae are smaller on the sides of the laesura. K. Equatorial view. The verrucae are low and mainly rounded; their surfaces are micro-sculptured. Globules of different sizes are fused to the perispore surface and are more evident on the left. L. Detail of the equatorial surface showing obscure verrucae shape due to the thick perispore; the perispore surface is micro-verrucate and perforated (arrow head). M. Fracture across the sporoderm. A different zone in the exospore toward its inner surface can be appreciated. Scale bars: A, B, E, F, G, J, K: 10 μm; C, D, H, L and M: 3 μm; I: 2 μm

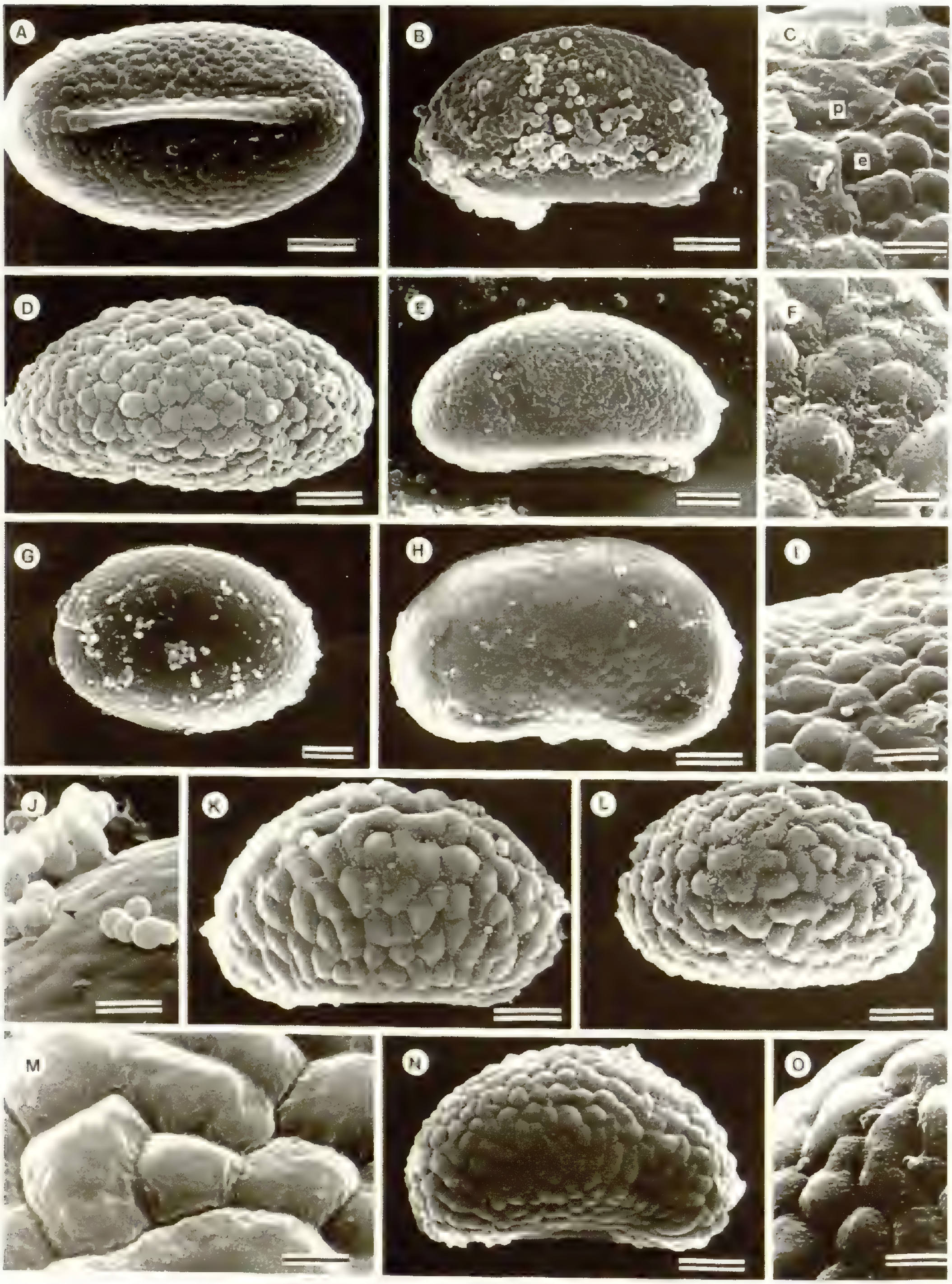

FIG. 5. SEM micrographs of *Polypodium* spores.
A–C, *Polypodium argentinum* (*Hurrel 51*) A. Proximal view, the laesura is a straight, thick ridge. The size and shape of ornamentation elements are uniform. B. Equatorial view, showing numerous globules attached to the perispore surface. C. The surface in detail with abraded perispore evident on the left and scattered globules of varied sizes fused to the perispore surface. Perforations are also

and so could be classified as "captive type" (sensu Lugardon, 1981). When observed with SEM, these globules seem to be sculptural elements. (Fig. 5 F; Fig. 6 D). In both species exospore ornamentation is verrucate. The verrucae are heterometric and located on a lower level than the globules. (Fig. 5 F; Fig. 6 D)

Discussion and Conclusions

The large spore size in *Campyloneurum tucumanense*, together with 32 or 64 spores per sporangium, and the greater plant size are characteristics probably related to polyploidy (Tryon & Lugardon, 1991). Walker (1985) detected polyploids in Jamaica and diploids among the South American species of this genus. Cytological studies are needed in order to determine if *C. tucumanense*, apparently endemic to the northwest of Argentina, is a polyploid.

Variations from 49 μm up to 95 μm in spore size were observed within the genus *Polypodium*. According to Tryon & Tryon (1982), the *Polypodium* species from America are diploid or tetraploid with relatively stable chromosome numbers. Further cytological studies of the material from Argentina would explain if the size differences are associated with differences in ploidy levels.

Phlebodium pseudoaureum has the smallest spores among the Polypodiaceae analyzed in this work and its tuberculate exospore is the thickest. These features, together with its greater plant size and pinnatifid glabrous lamina with anastomosing venation, differentiate it from other species in the study area.

The general features of the exospore in the species analyzed are in agreement with those of the "Polypodium vulgare" type as described by Hennipman (1990).

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present on the perispore (arrow head). The exospore is exposed on the right. The verrucae are different in size and shape, laterally fused and relatively smooth surface. Perforations are evident mostly at the junctions between the verrucae. D–F. *Polypodium bryopodum* (D: *Venturi 4802*. E–F: *Tur & Kiesling 18353*) D. Distal view with verrucate ornamentation. The verrucae are heterometric, mainly poligonal in outline, densely packed and laterally fused. E. Equatorial view with globules on the perispore (arrow head). F. Detail of a spot of the spore in figure E showing a micro-verrucate surface of the verrucae and perforations. G–J. *Polypodium chrysolepis* (*Castillo 35*) G. Distal view with single or grouped globules off different sizes. H. Equatorial view, the verrucae are low and apparently the perispore constitute a thin cover that makes their shapes slightly evident. There are a few scattered globules attached to the surface. I and J. Surface details. In I the characteristic of the verrucae are more evident than in J. J. masses of globules on the perispore surface, some of them are fused to the perispore (arrow head). The perispore surface is fairly smooth. K–M. *Polypodium lasiopus* (*Hawkes et al. 3976*) K. Equatorial view, the verrucae are tangentially elongated at the equatorial zone, forming true ridges. Small granules are present mainly at the junction places. L. Distal view, granules are present between sculpture elements. M. Detail of the surface showing irregular sizes and polygonal verrucae, mostly fused forming ridges. The perispore surface seems to be fairly smooth to scabrate. N–O. *Polypodium loriceum* (*Martínez* 641 *et al.*). N. Equatorial view, the verrucae are numerous per area unit, round in shape and low, but there is a general tendency to verruca fusion in the form of ridges. O. Detail of the surface, showing fused verrucae in the form of ridges. The perispore surface is rugulate. Small-sized globules are attached to the perispore surface in some places (arrow). Scale bars: A, B, D, E, G, H , K, L, N: 10μm; C, F, I, J, M and O: 3 μm.

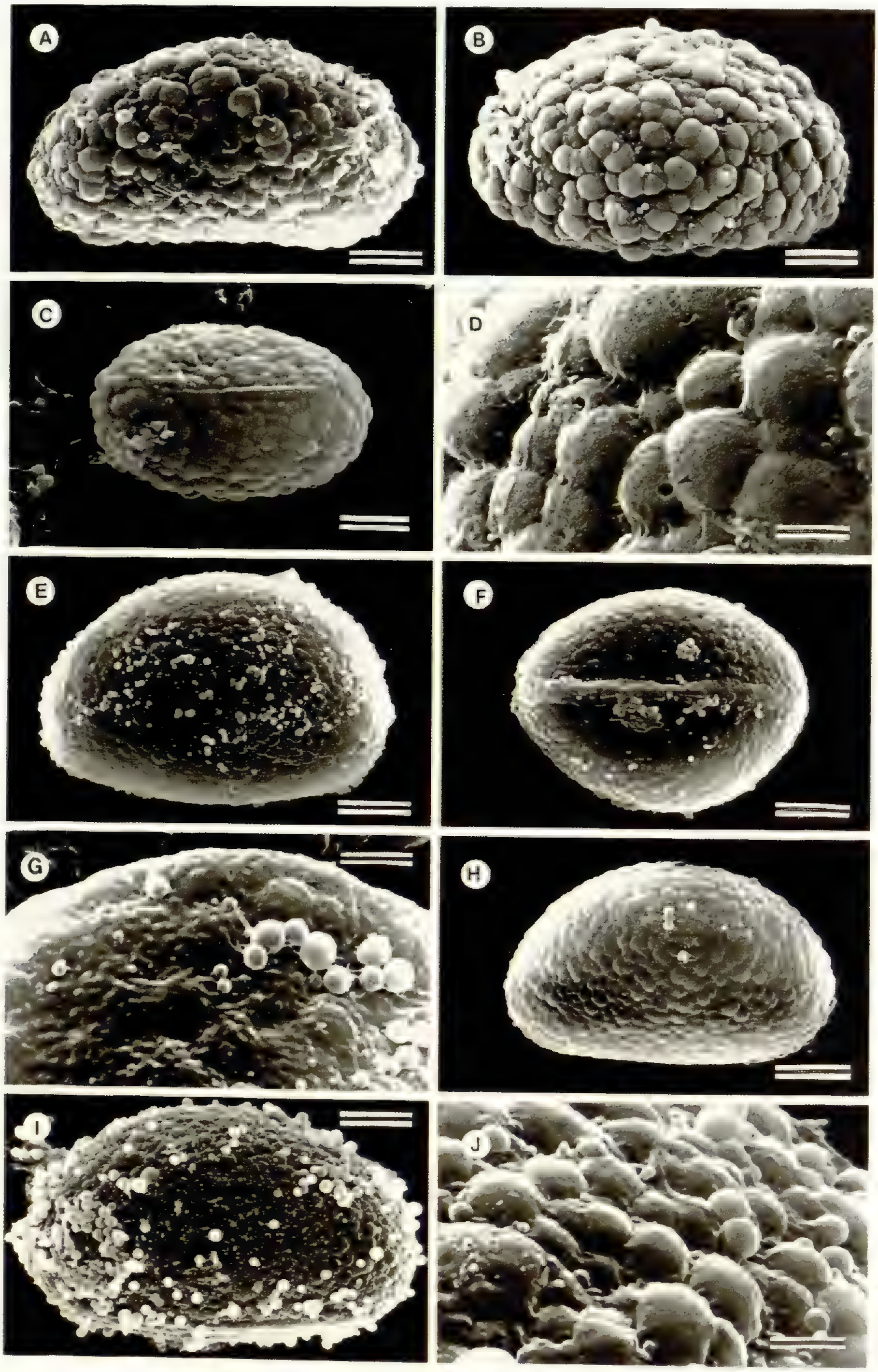

FIG. 6. SEM micrographs of *Polypodium* spores.
A–D, *Polypodium pleopeltidis* (*Cabrera, Kiesling & Zardini* 24008). A. Equatorial view, the verrucae are smaller toward the proximal pole. Scattered globules can be appreciated at different places B. Distal view, the surface is uneven and it has verrucae of different sizes and shapes in different levels. The large verrucae are laterally fused but each of them keeps the original outline.

Variations in sporoderm thickness, size of sculptural elements, number of perforations, and presence of granular material on spore surfaces were not considered to be as diagnostic characteristics because, they may be related to different stages of development (van Uffelen, 1992).

Perforations observed in the perispore and exospore of the analyzed spores could be associate with microchannels traversins the exospore; such structures were described by Lugardon (1974), van Uffelen (1992) and Hennipman (1990). The latter author classified the channels according to their size, shape and location within the exospore.

In all of the analyzed taxa the spore surface is covered with irregularly distributed globular bodies with a smooth or micro-verrucate surface, according to the taxa. These globules were acetolysis resistent and showed variable densities among spores of the same taxa. With LM they showed a density similar to that of the sporoderm. In some samples, *Polypodium bryopodum* and *P. pleopeltidis*, these globules were mistaken for elements of the exospore ornamentation. According to Lugardon (1981), these sporopollenin globules are usually present in the Filicopsids, and they were described as having a smooth surface and the same structure as the sporoderm. The globules were also analyzed using TEM in other taxa of Polypodiaceae (van Uffelen & Hennipman, 1985; Hennipman, 1990; van Uffelen; 1993 and Tryon & Lugardon, 1991).

We would noted in our analysis that the spores of some *Polypodium* species from the study area (*P. argentinum*, *P. squalidum* and *P. chrysolepis*) have similarities to those of *Pleopeltis*, some others (*P. bryopodum* and *P. pleopeltidis*) are similar to those of *Pecluma*, and others, such as *P. lasiopus* and *P. loriceum*, to those of *Microgramma*. These observations are in agreement with de la Sota (1977), who suggested that *Polypodium* is not a well delimited genus, and that certain taxa are closer to *Microgramma* whearas some others are closer to *Pleopeltis*. Later systematic studies grouped several species of *Polypodium* within *Pecluma* (Price, 1983), while others species were transferred to *Pleopeltis* (de la Sota, *in press*) and the species *Polypodium*

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Small, single globules attached to the surface can be seen in several places (arrow head). C. Proximal view. The laesura is marked by a straight ridge. The verrucae are small on both sides of the laesura. D: Detail of the surface. The verrucae are heterometric and the spaces between them are masked possibly by the perispore. Perforations of different sizes are located mainly on and between the verrucae (arrow heads). The perispore is micro-verrucate. E–G, *Polypodium squalidum* (*Zuloaga & Deginami 327*). E. Equatorial view, the verrucae are numerous per area unit, uniform in shape and size. Scattered single and grouped globules are attached to the surface. F. Proximal view. The laesura appears as a stick ridge. There are also globules at the proximal pole. G. Detail of the surface. The limits between the verrucae of the exospore below are obscure. The perispore surface is rugate and perforations are evident (arrow heads). Single and grouped globules of different sizes with a smooth surface are fused to the perispore. H–J, *Polypodium tweedianum* (*Palací 104*). H and I. Equatorial views. The presence of globules is variable. Some verrucae are fused, forming short ridges. J. Detail of surface. Verrucae are clearly defined although laterally fused. Globules of different sizes are fused to the surface. The perispore surface is relatively smooth, although some perforations are present (arrow heads). Scale bars: A, B, C, E, F, H, I: 10 μm; G, D and J: 2 μm.

chrysolepis was considered as *Microgramma* by Tryon & Stolze 1993 based on some venation characteristics.

The spores of *Polypodium loriceum* and *P. lasiopus* differ from other studied *Polypodium* species in exospore ornamentation. In the former species the verrucae are generally fused and form radial ridges proximally. These species belong to the "*Polypodium-loriceum* group" that is represented by three species in Argentina (de la Sota, Giudice & Gaute, *pers.comm.*). Our observations suggest that spores are systematic of value at infrageneric level.

Within *Pecluma*, *P. fillicula* is differentiated from other species on the basis the small size and a pubescent-scaly rachis. The thin exospore, low verrucae and smooth perispore allow it to be differentiated from others species within the genus.

Concluding Remarks

Five of the genera of Polypodiaceae from Northwestern Argentina, *Campyloneurum*, *Microgramma*, *Pecluma*, *Pleopeltis* and *Polypodium*, have verrucate spores, whereas the spores produced by *Phlebodium* are tuberculate. Within the verrucate spores taxa, variations were observed in verrucae size, shape, surface pattern and degree of fusion. The large size, together with other characteristics of *Campyloneurum tucumanenese* spores, may be related to polyploidy. In all the taxa studied the exospore was the thickest wall layer. As revealed by LM it is apparently double-layered and shows wide variation in ornamentation and structure. The inner exospore layer is thin, generally 1/3 to 1/6 the thickness of the outer layer. In acetolyzed material, as observed with LM, the outer exospore appears lighter than the inner one and, apparently, the verrucae or tubercles are restricted to the outer exospore layer.

In all cases the perispore is difficult to distinguish and measure using LM because it is thin and adhered to the exospore. However, it was possible to distinguish it in fractured spores with SEM. It is micro-ornamented, generally perforated and covered without any modification to exospore ornamentation.

The presence of sporopollenin globules is frequent on most of the spores analyzed. They are single or grouped and are either attached or fused to the perispore surface.

We noticed that there is little systematic agreement between systematists and palynologists when considering the significance of spore characteristics in the Polypodiaceae. Nevertheless, in the taxa analyzed here, palynological characteristics, together with morphological data, allow us to identify some genera such as *Pecluma* and *Phlebodium*, as well as recognize species groups within the genus *Polypodium*, e.g. the *Polypodium-loriceum* group.

We are continuing this study on the spore wall of the Polypodiaceae with TEM analyses to determine the structure and stratification of the spore wall, the relationship between perforations and channels within the exospore, and to characterize the globules.

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Spore Viability Under Different Storage Conditions in Four Rupicolous *Asplenium* L. Taxa

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ABSTRACT.—Spore germination of four rupicolous taxa of *Asplenium* (*A. adiantum-nigrum*. var. *adiantum-nigrum*, *A. adiantum.nigrum*. var. *silesiacum*, *A. septentrionale* subsp. *septentrionale* and *A. ruta-muraria*. subsp. *ruta-muraria*) was determined after 1, 6, and 12 months of storage in Eppendorf tubes (dry storage) or on agar plates (wet storage) at –20, 5 and 20°C. In general, technique and temperature factors and the moisture-temperature interaction, had a significant effect on germination percentage. In all cases, except for *A. ruta-muraria*, germination percentage was maintained in wet and dry storage, but in the dry storage method percent germination was higher. These results indicate some capacity of *Asplenium* spores to withstand desiccation, and that ecological requirements of species may influence spore viability and should be taken into account when designing spore conservation programs. Spores of *A. ruta-muraria* yielded better results in wet storage. In dry storage its response was different from that of the other three taxa. Wet storage at –20°C killed all or most spores of all taxa.

Interest in the conservation of pteridophyte spores has become evident in recent decades, because they are easy to obtain, can be stored in large quantities, and can germinate rapidly in simple media (Dyer, 1979). Spores are of interest not only in *ex situ* conservation programs, but also, as Page *et al.* (1992) show, in taxonomic studies in the broadest sense, and as a commercial source in horticulture. However, in contrast to seed conservation (Baskin and Baskin, 2001 and references therein), little is known about the factors that affect spore viability during storage.

Lloyd & Klekowski (1970) calculated the variation in viability of chlorophyllous (green) and non-chlorphyllous spores over storage periods of 2 months to 3 years, noting the marked contrast between *Equisetum* (12 to 24 days viability) and *Asplenium* (up to 48 years).

The conditions under which spores are stored have a notable impact on their viability. Generally, to avoid deterioration, they are stored in dry, ambient or low temperatures, although in some cases this has resulted in loss of viability (Beri and Bir, 1993; Camloh, 1999). Another option that has been tried is storage of spores in a hydrated state (Lindsay *et al.*, 1992), analogous to conditions prevailing in natural spore banks in which spores of some species can remain viable for long periods (Lindsay and Dyer, 1990). It has been

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observed that this type of storage may be more effective than dry storage for certain species.

Pteridophyte spores may remain viable, in a metabolically inactive state, when conditions are not adequate for germination (Page, 1979). The length of time over which spores can maintain viability varies enormously from species to species (Miller, 1968) and it has been shown that other characters, such as spore age (Raghavan, 1989 and references therein), ploidy level (Kott & Peterson, 1974; Kott & Britton, 1982), and the presence of chlorophyll are influential. Even though they can survive desiccation (Lloyd and Klekowsky, 1970; Lebkuecher, 1997), chlorophyllous spores have generally limited viability, compared with pteridophytes with much longer-lived non-chlorophyllous spores.

Page *et al.* (1992) point out the need to investigate storage conditions that guarantee the maintenance of spore viability for the longest possible time, their genetic integrity, and their developmental capacity. Having available collections of adequately stored spores is of interest in order to avoid the loss of species in nature, in the case of threatened species, while at the same time offering the possibility of having subsequent developmental phases with which to investigate other aspects of the biology of the species.

In the present study, various storage conditions were tested on the spores of four *Asplenium* taxa: *A. septentrionale* (L.) Hoffm. subsp. *septentrionale*, *A. ruta-muraria* L. subsp. *ruta-muraria*, *A. adiantum-nigrum* L. var. *adiantum-nigrum*, and *A. adiantum-nigrum* var. *silesiacum* (Milde) Viane & Riechstein. These taxa constitute a homogeneous biogeographical and ecological group. They are circumboreal species (Pichi Sermolli *et al.*, 1998) orophyllous, rupicolous, and all are tetraploids. *Asplenium septentrionale* subsp. *septentrionale* is an autotetraploid derived from subspecies *caucasicum* Fraser-Jenkins & Lovis (Lovis, 1964) and is widely distributed throughout Eurasia as well as disjunctly in North Africa and on the Pacific coast of North America, where it preferentially inhabits acid substrates. *Asplenium ruta-muraria* subsp. *ruta-muraria* is also an autotetraploid but derived from subspecies *dolomiticum* Lovis & Reichst. (Lovis, 1964) and is distributed across a broad belt in the northern hemisphere, in Europe, Asia and America, having its southern limit in North Africa. It prefers basic substrates. Finally, *A. adiantum-nigrum* is an allotetraploid arising from crossing and subsequent chromosomal duplication of *A. cuneifolium* Viv. and *A. onopteris* L. (Shivas, 1969). It has a wide range throughout Europe, Macaronesia, Asia, Africa, North America and Australia, where it colonizes cracks and fissures preferentially in siliceous rocks. Two varieties are recognized: the typical variety described above, and the variety *silesiacum* (serpentinicolous ecotype), of ultrabasic substrates in northern and western Europe (Salvo, 1990).

Different degrees of hydration (wet and dry) have been combined with different temperature regimes in order to analyse the percentage germination after varying periods of storage. This same methodology has been used in a previous study (Quintanilla *et al.*, 2002) of a group of relict Macaronesian species that inhabit forest floors. These authors aimed to optimise the method

TABLE 1. Location of populations studied, collector and date of collection.

Taxa	Location	Altitude	Coordinates UTM	Collection data	Collector(s)
A. septentrionale	Madrid. Manzanares El Real. La Pedriza	1300	30TVL2310	July 2000	C. F. Aragón & R. G. Camacho
A. adiantum-nigrum var. *adiantum-nigrum*	A Coruña. A Baña.	225	29TNH2153	October 2000	L. G. Quintanilla
A. adiantum-nigrum var. *silesiacum*	A Coruña. Sierra de la Capelada. Chao do Monte	280	29TNJ8139	October 2000	J. Amigo & L. G. Quintanilla
A. ruta-muraria	Guadalajara. Somolinos.	1000	30TVL9263	July 2000	E. Pangua & S. Pajarón

of storage of viable spores as part of a conservation strategy. Although the taxa included in this study present no problem from a conservation point of view, our objective was to establish whether the optimal spore storage method varies among the taxa.

MATERIAL AND METHODS

Spores were obtained from populations of each taxon (Table 1). Fertile fronds of 15 sporophytes were collected per population and transported to the laboratory where they were washed under tap water and pressed for two weeks until the spores were released. Each sample was obtained from a mixture of spores from all sporophytes gathered in each population.

The spores of these four taxa were subjected to different storage conditions in which different degrees of hydration (wet and dry techniques) and temperatures were combined. With the wet technique, spores were sown directly on a mineral agar medium (Dyer, 1979) that had been sterilized in an autoclave at 20 atm and 125°C for 20 minutes, on sterile plastic Petri dishes (5.5 cm diameter), which were sealed with Parafilm (American National Can, Chicago) to avoid desiccation. To prevent contamination, the antifungal agent Nystatin (100 U ml^{-1}) was added to the culture medium after autoclaving and also, in all sowings the spore samples were passed through two layers of lens cleaning tissue (Whatman International Ltd. Maidstone, n° 2105841) to eliminate impurities, remains of sporangial walls, etc. With the dry technique, spores were kept in Eppendorf tubes until germination tests were carried out at which time they were plated out as above. The dishes and tubes were stored at temperatures of 20, 5 and −20°C. They were kept in the dark by wrapping them in aluminium foil to avoid germination during the storage period.

Germination tests were carried out after 1, 6 and 12 months of storage. All dishes were incubated for 30 days in a culture chamber at 21°C and 30 μmol m^{-2} s^{-1} intensity of photonic flow, with a 16 h light: 8 h dark photoperiod. Four replicates were incubated for each combination of technique and temperature

and the percentage germination was assessed after 30 days. This same germination test was carried out before storage to establish a control group. Germination was considered to have occurred if the spore wall was broken and the first rhizoid had emerged. Germination rate (%) was calculated on the basis of a count of 100 randomly chosen spores from each dish.

To determine the effects of technique (wet and dry) and temperature (20, 5 and –20°C) on germination rate, the percentages were arcsine-transformed and their means compared by a two-way analysis of variance (Zar, 1999). The analyses were repeated for 1, 6 and 12 months of storage. The multiple comparisons among means for the identification of homogeneous groups, wherein the effect of a factor was significant ($p < 0.05$), were made using the Tukey test ($p < 0.05$). All analyses were done with the SPSS statistical program (1999).

Results

As in the majority of pteridophyte species, spores of the taxa studied required the presence of light to germinate. The effect of technique and temperature factors, and their interaction, upon spore germination was statistically significant in all cases, except for the hydration factor after one month of storage in the case of *A. ruta-muraria* (F = 0.059; Table 2). The existence of a significant interaction between factors implies that the effect of each is different for each level. Thus, multiple comparisons between media were made for each possible combination of hydration and temperature.

The response of *A. adiantum-nigrum* var. *adiantum-nigrum*, *A. adiantum-nigrum var. silesiacum* and *A. septentrionale* to the different storage conditions was similar (Fig. 1A, B and C), whereas those of *A. ruta-muraria* were more variable (Fig. 1D). In general, high percentages of germination were found in the first three taxa, irrespective of the storage conditions, except for wet storage at –20°C, in which case only a small percentage of spores of these three taxa germinated after the first month. In these three cases, dry storage was fairly effective, although there was a slight decrease in the percentage of viable spores after 12 months of storage when kept at –20°C (Fig. 1A, B and C). Dry storage at 5°C (Table 3) was significantly higher ($p < 0.05$) than for dry-storage at 20 and –20°C, in the case of *A. adiantum-nigrum* var. *silesiacum*, and at –20°C for *A. septentrionale*, after 12 months storage (see Table 3).

With respect to *A. ruta-muraria*, the best results were obtained with wet storage at 20 and 5°C; no spores germinated at –20°C. Percentages achieved with the dry technique were generally lower than under humid conditions and lower than in the other taxa, except for the results after 6 months' storage at 5 and –20°C (Fig. 1D), where similar percentages were obtained to those with wet storage (Table 3).

Discussion

Our results indicate that for the taxa studied, except *A. ruta-muraria*, storage under any of the tested conditions allowed relatively high percentages of viable spores, except with wet storage at –20°C. Under those conditions there

TABLE 2. Levels of significance of the effects of technique and temperature factors on percentage spore germination after 1, 6 and 12 months of storage. MS, mean squared; df, degrees of freedom; *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.001$.

			Storage time					
			1 month		6 months		12 months	
Taxa	Source of variation	d.f.	MS	*F*	MS	*F*	MS	*F*
A. septentrionale	Technique	1	2,303,118	91,188***	1,583,659	77,284***	849,024	23,600***
	Temperature	2	2,167,125	85,803***	3,168,146	154,609***	3,727,413	115,463***
	Technique × Temperature	2	1,945,107	77,013***	1,829,107	89,262***	1,503,217	46,565***
	Error	18	25,257	...	20,491	...	32,282	...
A. adiantum-nigrum var. adiantum nigrum	Technique	1	1,983,658	88,865***	2,361,546	214,497***	1,960,820	207,121***
	Temperature	2	2,513,572	112,603***	3,809,133	345,977***	3,316,035	350,271***
	Technique × Temperature	2	1,824,572	81,737***	2,859,518	259,725***	2,600,584	274,695***
	Error	18	22,322	...	11,010	...	9,467	...
A. adiantum-nigrum var. silesiacum	Technique	1	2,963,416	75,106***	3,358,753	126,386***	2,335,769	207,865***
	Temperature	2	2,506,259	64,104***	2,917,486	109,782***	2,674,307	237,922***
	Technique × Temperature	2	1,605,239	41,058***	1,899,082	71,460***	1,775,800	158,032***
	Error	18	39,097	...	26,575	...	11,237	...
A. ruta-muraria	Technique	1	15,918	0.059 ns	455,188	8,684**	2,263,204	22,964***
	Temperature	2	1,977,344	7,371**	1,941,044	37,032***	3,208,584	32,557***
	Technique × Temperature	2	1,305,054	4,865*	2,477,879	42,274***	1,491,976	15,139***
	Error	18	268,264	...	52,415	...	98,553	...

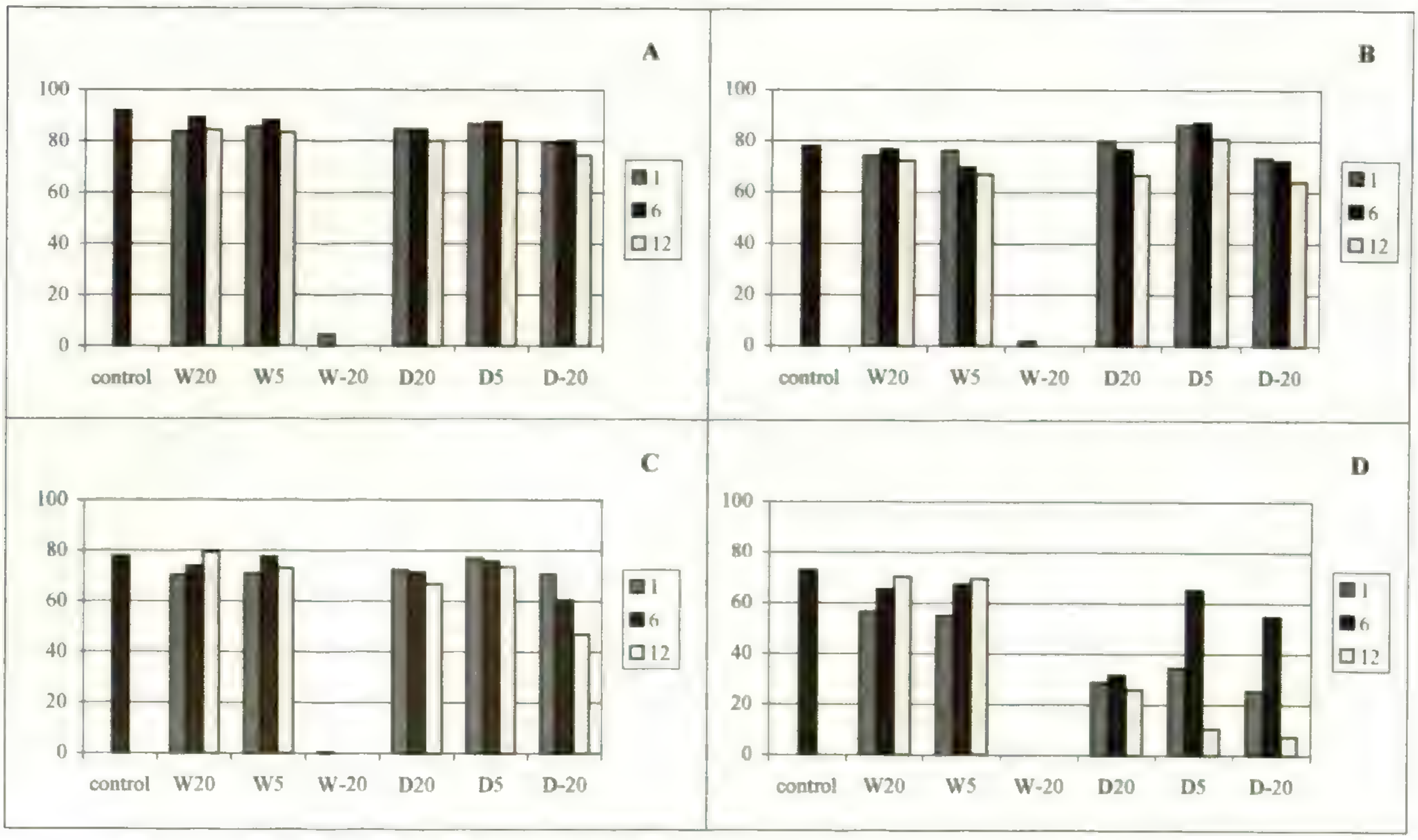

FIG. 1. Germination percentage after 1, 6, and 12 months' storage with different techniques (W, wet; D, dry) at temperatures of 20°, 5°, and -20°C. (A) *Asplenium adiantum-nigrum* var. *adiantum-nigrum*; (B) *A. adiantum-nigrum* var. *silesiacum*; (C) *A. septentrionale*; (D) *A. ruta-muraria*.

was practically no germination after one month of storage. This combination of hydration and temperature was generally inefficient at maintaining spore viability, confirming observations under identical conditions by Quintanilla *et al.* (2002) on relict forest species. This implies that this combination is not efficient irrespective of species ecology. Pangua *et al.* (1999) also noted a decrease in germination for spores of *Cryptogramma crispa* (L.) R. Br. kept in the wet at –18°C. Germination percentage in that species varied among populations. In this species wet spores subjected to a temperature of 70°C yielded higher germination percentages than did dry spores when subjected to the same temperatures after 24 h of treatment (Simpson and Dyer, 1999). Given that a dry –20°C treatment did not result in such drastic reduction in germination, it is obvious that previous hydration renders the spores more sensitive to freezing.

Hill (1971) showed that spores of *Adiantum pedatum* L. and *Thelypteris palustris* Schott, after a month of freezing in liquid medium, had higher percentages of germination for the characteristic periods of time than when spores were kept at ambient temperature. Although it has not been shown that longer preservation times yielded the same results, it nevertheless appears that these spores may require chilling in order to germinate. Hill (1971) did not specify whether the spores were frozen immediately after their inclusion in the medium or if there was a time of imbibition.

Wet storage at 20 and 5°C maintained the viability of a large number of spores of all taxa studied and hence represents an effective method of storage.

TABLE 3. Percentage germination (mean ± standard error) of spores without previous storage (control) and after 1, 6, and 12 months storage, with wet (W) and dry (D) techniques at temperatures of 20°, 5° and −20°C. The vertical lines indicate those groups with homogenous means, between which there are no significant differences.

		Storage time					
		1 month		6 months		12 months	
Taxa	Control	Germination	Treatment	Germination	Treatment	Germination	Treatment
A. septentrionale	77.8 ± 3.1	0.5 ± 0.5	W −20	0.0 ± 0.0	W −20	0.0 ± 0.0	W −20
		70.2 ± 1.5	W 20	60.5 ± 6.8	D −20	47.0 ±8.7	D −20
		70.7 ± 5.8	D −20	71.5 ± 3.6	D 20	66.7 ± 5.7	D 20
		71.0 ± 5.5	W 5	74.0 ± 1.4	W 20	73.0 ± 1.6	W 5
		72.5 ± 4.0	D 20	76.0 ± 3.4	D 5	73.5 ± 4.4	D 5
		77.0 ± 3.1	D 5	77.5 ± 2.6	W 5	79.2 ± 1.7	W 20
A. adiantum-nigrum var. *adiantum nigrum*	92.0 ± 1.1	4.5 ± 1.9	W −20	0.0 ± 0.0	W −20	0.0 ± 0.0	W −20
		79.5 ± 4.1	D −20	79.7 ± 3.1	D −20	74.5 ± 1.6	D −20
		84.0 ± 0.7	W 20	84.7 ± 1.8	D 20	80.0 ± 2.0	D 20
		84.7 ± 2.4	D 20	87.7 ± 2.9	D 5	80.5 ± 3.0	D 5
		85.7 ± 2.6	W 5	88.2 ± 1.0	W 5	83.2 ± 2.3	W 5
		86.7 ± 1.1	D 5	89.2 ± 1.5	W 20	84.5 ± 2.1	W 20
A. adiantum-nigrum var. *silesiacum*	79.0 ± 0.6	2.0 ± 1.7	W −20	0.0 ± 0.0	W −20	0.0 ± 0.0	W −20
		74.2 ± 3.5	W 20	69.7 ± 6.4	W 5	64.2 ± 1.2	D −20
		73.5 ± 7.2	D −20	72.5 ± 3.7	D −20	66.7 ± 2.1	D 20
		76.2 ± 2.8	W 5	76.7 ± 3.9	D 20	67.0 ± 2.8	W 5
		80.2 ± 2.3	D 20	76.7 ± 3.6	W 20	72.5 ± 2.6	W 20
		86.2 ± 2.5	D 5	87.2 ± 2.7	D 5	81.0 ± 4.1	D 5
A. ruta-muraria	73.0 ± 2.6	0.0 ± 0.0	W −20	0.0 ± 0.0	W −20	0.0 ± 0.0	W −20
		25.5 ± 17.5	D −20	31.7 ± 11.5	D 20	7.2 ± 4.7	D −20
		28.7 ± 6.1	D 20	54.7 ± 6.8	D −20	10.5 ± 7.4	D 5
		34.5 ± 17.1	D 5	65.2 ± 3.3	D 5	26.0 ± 10.7	D 20
		55.0 ± 10.4	W 5	65.5 ± 1.0	W 20	69.5 ± 3.2	W 5
		56.5 ± 13.2	W 20	67.2 ± 5.1	W 5	70.5 ± 1.8	W 20

Lindsay *et al.* (1992) studied the response to spore hydration of four species with non-chlorophyllous spores and one with chlorophyllous spores, all of which were hygrophilous. Fully hydrated spores were capable of germinating at ambient temperature after two years of storage at 20°C at much higher percentages than those preserved dry but under otherwise identical conditions. Other hygrophilous species, such as *Woodwardia radicans* (L.) Smith and *Culcita macrocarpa* C. Presl., show a marked sensitivity to desiccation, such that only those spores that had been maintained in a wet medium germinated after 12 months' storage, 60% and 84% respectively, compared with those kept in the dry, 1% and 0% respectively, at the same temperature (Quintanilla *et al.*, 2002). These results are interesting because spores of natural spore banks would be in a wet state (Page *et al.*, 1992), especially those from species that inhabit places where the soil is very wet throughout the entire year.

Dyer and Lindsay (1992) have shown the persistent presence of *A. adiantum-nigrum*, *A. ruta-muraria* and *A. septentrionale* (L.) Hoffm. in British spore banks. However, in the latter two strictly rupicolous species, the presence of spores in these banks is of low importance, because, although the gametophyte is already established, new sporophytes cannot be established, possibly due to a problem of competition with other species. Furthermore, these two species require a minimum temperature for germination (Young, 1985; Pangua *et al.*, 1994; Dyer and Lindsay, 1996), which they may encounter within cracks or other protected places. Nevertheless, they can tolerate temperatures of up to 70°C for at least 24 h (Simpson and Dyer, 1999). These results may represent an adaptive efficiency in these species for the media they inhabit – exposed rocks with large temperature differences throughout the day and the year.

In our study, for *A. septentrionale*, *A. adiantum-nigrum* var. *adiantum-nigrum* and *A. adiantum-nigrum* var. *silesiacum* dry storage gave results similar to those with wet storage, although perhaps longer-term storage would have revealed greater differences. Nevertheless, in light of the results, it appears that these rupicolous taxa, have a relatively high capacity to withstand desiccation. Therefore it appears that ecological requirements of species can indeed result in taxa specific adaptations in terms of spore viability, although it must be born in mind that few species have been studied and considerable variability may exist in this respect.

In *A. ruta-muraria* wet storage, with the general exception of –20°C for all the taxa studied, was significantly more effective than dry in maintaining spore viability. Results obtained at 5 and 20°C are essentially the same, showing a germination capacity that increased slightly with time of storage. However, the response obtained with dry storage at different sampling times is difficult to explain. Only after 6 months of storage at 5°C was the germination comparable to that of wet storage. These results might be explainable by a need to go through a cold period before germination. For *A. ruta-muraria*, despite not being a hygrophilic species, the most suitable preservation method is wet at 5 or 20°C.

In *Polystichum setiferum* (Forsskål) Woynar and *Athyrium filix-femina* (L.) Roth, typical of wet woodlands, it has been observed that dry storage at 4°C results in increased spore viability after 12 and 24 months, respectively, whereas at 20°C, spore viability is practically lost (Lindsay and Dyer, in Simpson and Dyer, 1999). Spores of *Cyathea delgadii* Sternb. (Simabukuro *et al.*, 1998) and *Pteridium aquilinum* (L.) Kuhn remain viable for years after dry storage at 4°C (Ashcroft and Sheffield, 2000), for which reason the authors have proposed the routine use of this storage technique and temperature. Some species with chlorophyllous spores, such as those of *Osmunda*, also retain their viability after years of dry storage at temperatures of 2° and 6°C (Stokey, 1951). In our case, therefore, it appears that the dry technique would be a good option for the conservation of the spores since, although the two techniques have yielded favourable results, the dry technique has some advantages, such as saving space, time and materials. The ideal storage temperature, in this case, would be 5°C, bearing in mind the results of our experiments, where the spores kept dry at this temperature had somewhat higher germination percentages in *A. adiantum-nigrum* var. *adiantum-nigrum*, and significantly higher percentages in *A. adiantum-nigrum* var. *silesiacum* and *A. septentrionale*.

Recent studies (Agrawall *et al.*, 1993; Pence, 2000) have demonstrated the effectiveness of preservation of dried chlorophyllous and non-chlorophyllous spores at –196°C in liquid nitrogen. Pence (2000) observed germination rates of spores of *A. ruta-muraria* stored under these conditions that were similar to that of the control population. These results may imply that imbibed spores are affected by very low temperatures, but that keeping them dry is a good conservation technique.

The time of storage and the processes of sterilization bring about alterations in germination and subsequent development of the gametophytes. Smith and Robinson (1975) studied germination of *Polypodium vulgare* L. using spores dry-stored at 4°C for 7 years. They observed a decrease in germination and an increase in the proportion of abnormal gametophytes. Similar results were obtained by Beri and Bir (1993) for *Pteris vittata* L., stored at room temperature for 100 days; spores lost germination capacity in association with total loss of sugars, amino acids and proteins. Camloh (1999) observed in *Platycerium bifurcatum* (Cav.) C. Chr. that sterilized spores lost viability and that with age there were fewer and shorter rhizoids. It would be interesting, in addition to germination studies for conservation, to study the impact on the development of the gametophyte. In our work, although we have not carried out a thorough post-germination study, the plates used in the various experiments remained in culture chambers at 20°C for 6 months and the gametophytes appeared to develop normally. This suggests that storage time may not affect the subsequent development of the prothalli.

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A Comparison of Useful Pteridophytes between Two Amerindian Groups from Amazonian Bolivia and Ecuador

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ABSTRACT.—An ethnobotanical study of the pteridophytes used by the Tacana and Huaorani indigenous groups from Amazonian forests of Bolivia and Ecuador is presented. Twenty-four useful species, eleven for Bolivia and fourteen for Ecuador, are reported. The only species used by both groups is *Cyathea pungens*. Most of the recorded uses (76%) are medicinal. Whereas the Tacana use most medicinal pteridophytes by external administration, to heal wounds, swelling, boils, and as eyewash, the Huaorani use them by internal administration, mainly to cure diarrhea, stomachache, body-pain, toothache, and colds. Three species are recorded for veterinary use (12%), to heal wounds and to expel intestinal parasites of domestic animals. Tree-ferns were widely used by all the interviewed informants.

Ferns and lycophytes have been employed for a wide variety of uses all over the tropics: cosmetics, dyes, fibers, folklore, flavorings and foods, medicines, and other minor products (e.g., Sodiro, 1893; Copeland, 1942; May, 1979; Murillo, 1983; Schultes and Raffauf, 1990; Ortega and Diaz, 1993; Nwosu, 2002). Although in Amazonia, rural, mestizo, and indigenous people have also used pteridophytes for those purposes, medicinal uses were the most important category (e.g., Davis and Yost, 1983; Murillo, 1983; Boom, 1985; Bourdy *et al.*, 2000). There is little published about useful pteridophytes in the Bolivian Amazon. Fifteen medicinal species are reported for the Chácobo indigenous community (Boom, 1985) and four medicinal species for the Tacana ethnic group (Bourdy *et al.*, 2000). Information from Amazonian Ecuador is more complete. There are five indigenous groups known to use pteridophytes, mainly for medicinal purposes. The Quichua use 12 species (Alarcón, 1988; Marles *et al.*, 1988; Báez, 1998); the Cofán 10 species (Cerón *et al.*, 1994; Cerón, 1995); and the Huaorani (Davis and Yost, 1983; Cerón and Montalvo, 1998), the Shuar (Báez and Backevall, 1998; Bennett *et al.*, 2002), and the Siona-Secoya (Vickers and Plowman, 1984) use four species each.

In this study, I record and compare the uses of pteridophytes by two indigenous people from western Amazonia: the Tacana from Bolivia and the Huaorani from Ecuador to determine whether the uses, applications, and administration of ferns and lycophytes follow the same general pattern of utilization for the two indigenous ethnic groups.

METHODS

In Ecuador, fieldwork was carried out in Orellana province, from April 1997 to May 1998, near the Huaorani communities of Tiputini (0°36′S; 76°27′W) and

Dicaro (0°56′S; 76°12′W). The first community is located within the limits of the Yasuní National Park; the second is located within the Huaorani Ethnic Reserve. The study area is tropical evergreen rainforest at 200–300 m elevation. According to drainage and flooding, three broad forest types (*tierra firme*, floodpain, and swamp) can be recognized (for a specific description of the area, see Romero-Saltos *et al.*, 2001). The indigenous Huaoranis are hunters and fruit-gatherers, and were first contacted less than 50 years ago (Yost 1991; Cabodevilla 1994). They have a deep knowledge of the biology of the forests and their useful plants (Macía *et al.*, 2001)

Fieldwork in Bolivia was conducted in Abel Iturralde province, Departamento La Paz, from April 2001 to April 2002, in various areas of the Madidi National Park and in the Área Natural de Manejo Integrado Madidi. The study site is a transitional area between Amazonian forest and low montane forest from 260 to 1070 m elevation. Ethnobotanical information was obtained from five pilot-study remote areas (13°53′S–68°09′W; 14°10′S–67°54′W). The indigenous Tacanas were contacted in the 17th century by Franciscan missionaries (Wentzel, 1989; Hissink and Hahn, 2000) and today they are mainly farmers, although hunting and fishing are occasional activities.

Five male informants (>40 years old) were separately interviewed about useful pteridophytes in each of the two study sites; the participation of women was not possible. The informants were chosen by villagers as the most plant-knowledgable people within their own communities. In Bolivia, the informants came from three Tacana communities (Carmenpecha, Macahua, and Tumupasa), and in Ecuador from two Huaorani communities (Dicaro and Tiputini). A semi-structured interview was followed for ethnobotanical queries (Alexiades, 1996). All interviews and fern collections were conducted in the field with the informants. In this paper, I follow the taxonomic system of Tryon and Tryon (1982) for ferns and lycophytes. Vouchers from Bolivia have been deposited in LPB, MA, and MO, and vouchers from Ecuador in AAU, MA, QCA, and TUR.

Results

The generic vernacular name for pteridophytes is '*atarisi*' in the Tacana language whereas in the Huaorani language it is '*toyuba*'. Twenty-four pteridophytes were used by both groups: 11 species for the Tacana and 14 species for the Huaorani. The tree-fern *Cyathea pungens* was used by both. Most uses (76%) were for medicinal purposes. In Bolivia, four species were used to heal wounds and as an antiseptic (including two *Campyloneurum* species), and two more species as an anti-inflammatory for boils and swelling. In Ecuador, four species were used to alleviate diarrhea and stomachache (including two *Adiantum* species), three species to cure general bodypain (including two *Polybotrya* species), and two species to alleviate toothache. Three species were used for veterinary medicine (12%).

Adiantum humile Kunze [Pteridaceae]
Huaorani vernacular name: *Toyuba*.
Vouchers: *Macía et al. 874, 2841, 3388*.
Tierra firme, floodplain, and swamp (Ecuador).
USES: A decoction of crushed fronds is drunk to cure diarrhea and stomachache. One informant reports that this plant was only used by shamans as a medicinal remedy.

Adiantum obliquum Willd. [Pteridaceae]
Tacana vernacular name: *Atarisi*.
Voucher: *Macía et al. 5945*.
Tierra firme in high Amazonian forest (Bolivia).
USES: Crushed fronds are directly applied to stop hemorrhaging and heal wounds.

Adiantum platyphyllum Sw. [Pteridaceae]
Tacana vernacular name: *Cucubio ina*.
Voucher: *Macía et al. 4455*.
Tierra firme in high Amazonian forest (Bolivia).
USES: Some drops from crushed fronds are used as eyewash when vision is not clear.

Adiantum pulverulentum L. [Pteridaceae]
No vernacular name given.
Voucher: *Macía et al. 2899*. Tierra firme (Ecuador).
USES: A decoction of the fronds is drunk to cure diarrhea.

Alsophila cuspidata (Kunze) D. S. Conant [Cyatheaceae]
Tacana vernacular name: *Atarisi*.
Vouchers: *Macía et al. 4017, 4424, 6492*. High Amazonian and low montane tierra firme forests (Bolivia).
USES: Mucilage from the apical part of the cut stem applied to boils. A poultice made from this sap is externally applied to reduce swelling in any part of the body.

Bolbitis nicotianifolia (Sw.) Alston [Dryopteridaceae]
Huaorani vernacular name: *Acagueme*.
Vouchers: *Macía et al. 2861, 3218, 3668*. Floodplain and swamp (Ecuador).
USES: Boiled crushed rhizome with one pinna is drunk to cure stomachache; a decoction of the rhizome is drunk to alleviate body pain and fever ('*calentura*').

Campyloneurum fuscosquamatum Lellinger [Polypodiaceae]
Huaorani vernacular name: *Toyuba*.
Vouchers: *Macía et al. 1592, 2982*. Tierra firme, floodplain, and swamp (Ecuador).

USES: A decoction of the crushed fronds is drunk to cure colds and coughs.

Campyloneurum repens (Aubl.) C. Presl [Polypodiaceae]

Tacana vernacular name: *Atarisi.*

Voucher: *Macía et al. 5658.* Tierra firme in high Amazonian forest (Bolivia).

USES: Crushed fronds are applied directly to heal wounds, fronds may also be placed under bandages for several hours.

Campyloneurum sphenodes (Kunze ex Klotzsch) Fée [Polypodiaceae]

Tacana vernacular name: *Chati ina.*

Voucher: *Macía et al. 5174.* Low montane tierra firme forests (Bolivia).

USES: Crushed fronds are directly applied to stop hemorrhaging and heal wounds. When dogs have been bitten by wild animals, people chew the fronds and apply them to the dogs wounds.

Cyathea amazonica R. C. Moran [Cyatheaceae]

Tacana vernacular name: *Atarisi.*

Voucher: *Macía et al. 5635.* Tierra firme in high Amazonian forest (Bolivia).

USES: Crushed apical part of the stem is macerated in cold water or urine and applied directly to scabby dogs. According to our informant, it cannot be used on people because it is too "strong".

Cyathea delgadii Sternb. [Cyatheaceae]

Tacana vernacular name: *Atarisi.*

Vouchers: *Macía et al. 5357, 6183.* Low montane tierra firme forests (Bolivia).

USES: Mucilaginous sap from central apical part of the cut stem is directly applied to boils.

Cyathea lasiosora (Mett. ex Kuhn) Domin [Cyatheaceae]

Huaorani vernacular name: *Toyuba, toyuto.*

Vouchers: *Macía et al. 400, 655.* Tierra firme and swamp (Ecuador).

USES: Drops of mucilaginous sap, from the basal part of a cut pinna or apical part of the cut stem, are used to alleviate toothache, placing them directly on the gum. Five informants from two Huaorani communities reported this use. Well-dried stems are occasionally used for firewood.

Cyathea pungens (Willd.) Domin [Cyatheaceae]

Huaorani vernacular name: *Toyuba, toyuwe.*

Vouchers: *Macía et al. 309, 2441, 2721.* Swamp forest (Ecuador).

USES: Drops of mucilaginous sap from the basal part of a cut pinna are used to alleviate toothache by placing them directly on the gum.

Tacana vernacular name: *Atarisi.*

Voucher: *Macía et al. 4127.* Tierra firme in high Amazonian forest (Bolivia).

USES: Mucilaginous sap from central apical part of the cut stem is applied directly on skin in cases of swelling.

Equisetum giganteum L. [Equisetaceae]
Spanish vernacular name: *Bigote de tigre*.
Voucher: none. Floodplain in Amazonian forest (Bolivia).
USES: A decoction of crushed aerial stems and whorls of branches is drunk to alleviate kidney and bladder pain.

Lomariopsis japurensis (Mart.) J. Sm. [Dryopteridaceae]
Tacana vernacular name: *Chati ina*.
Voucher: *Macía et al. 3880*. Tierra firme in high Amazonian forest (Bolivia).
USES: Dried fronds are pulverized and put directly on wounds to heal them.

Melpomene melanosticta (Kunze) A. R. Sm. and R. C. Moran [Grammitidaceae]
Tacana vernacular name: *Atarisi*.
Voucher: *Macía et al. 6224*. Low montane tierra firme forests (Bolivia).
USES: Whole plant is used for womens' necklaces because their fresh rhizomes are fragant for a long time.

Microgramma fuscopunctata (Hook.) Vareschi [Polypodiaceae]
Huaorani vernacular name: *Guimipume*.
Vouchers: *Macía et al. 1535, 2995*. Swamp forest (Ecuador).
USES: Boiled fronds are rubbed on joints (knee, elbow, shoulder) to alleviate aching.

Polybotrya crassirhizoma Lellinger [Dryopteridaceae]
Huaorani vernacular name: *Toyuba, toyuba bengana*.
Vouchers: *Macía et al. 623, 684*. Tierra firme and floodplain (Ecuador).
USES: A decoction of croziers is drunk to alleviate body pain.

Polybotrya osmundacea Humb. & Bonpl. *ex* Willd. [Dryopteridaceae]
Huaorani vernacular name: *Toyuba*.
Vouchers: *Macía et al. 605, 3377*. Tierra firme forest (Ecuador).
USES: A decoction of croziers is drunk to alleviate body pain.

Saccoloma inaequale (Kunze) Mett. [Dennstaedtiaceae]
Huaorani vernacular name: *Toyuto*.
Vouchers: *Macía et al. 1521, 3389*. Tierra firme and swamp (Ecuador).
USES: Crushed rhizome is fragrant and used as deodorant.

Selaginella exaltata (Kunze) Spring [Selaginellaceae]
Huaorani vernacular name: *Toyume*.
Vouchers: *Macía et al. 311, 339*. Swamp forest (Ecuador).

USES: Crushed rhizome is macerated in cold water, mixed with *chicha* (traditional beverage made from cassava), and drunk to cure stomachache and diarrhea.

Selaginella geniculata (C. Presl) Spring [Selaginellaceae]
Huaorani vernacular name: *Toyuba*.
Voucher: *Macía et al. 2555*. Floodplain (Ecuador).
USES: Fronds are used in ceremonial forehead bands for adornment at traditional Huaorani feasts.

Selaginella parkeri (Hook. & Grev.) Spring [Selaginellaceae]
Huaorani vernacular name: *Toyotome*.
Voucher: *Yanez, Macía et al.* 2231. Tierra firme (Ecuador).
USES: A liquid decoction of crushed rhizomes is given to dogs to expel intestinal parasites.

Thelypteris macrophylla (Kunze) C. V. Morton [Thelypteridaceae]
Huaorani vernacular name: *Toyuba*.
Voucher: *Macía et al. 2980*. Tierra firme, swamp (Ecuador).
USES: A decoction of crushed rhizome is drunk to cure stomachache.

DISCUSSION

Seventy-six percent of the ferns and lycophytes discussed with informants were used as medicines, a number similar to that reported in previous Amazonian ethnobotanical studies (e.g., Davis and Yost, 1983; Boom, 1985; Bourdy *et al.*, 2000). Tree-ferns were well known to all informants, and their applications coincided with those cited in previous literature (Davis and Yost, 1983 for *Cyathea*; Bourdy *et al.*, 2000 for *Alsophila*). Both study groups used *Cyathea pungens* as an anti-inflammatory, although the local application was different. This use is a special and valuable resource in need of more study.

Because the Ecuadorian region is more uniform and lower in elevation than the Bolivian study area, with a wide altitude gradient and in transition to low montane forests, explain the fern flora of the both regions is different, with little species overlap.

There are clear differences in the use of pteridophytes in Bolivia and Ecuador. The medicinal and veterinary species used by the Tacanas were administered externally (except for *Equisetum giganteum*), whereas those used by the Huaoranis were administered internally (except for *Microgramma fuscopunctata*). This differentiated medicinal pattern seems to be exclusive to pteridophytes, because medicinal administration of other vascular plants is not as specific for these two Amerindian people (Davis and Yost, 1983; Cerón and Montalvo, 1998; Bourdy 1999; Bourdy et al., 2000). Other indigenous groups also show preferences for medicinal administration of ferns and lycophytes: the Chácobo from Bolivia and the Quichua from Ecuador, mostly administered their preparations internally (Boom, 1985; Alarcón, 1988; Marles

et al., 1988), whereas the Cofán from Ecuador administer their preparations externally (Cerón et al., 1994; Cerón, 1995).

The fragant rhizomes of two species are used as a perfume or a deodorant. Furthermore, the rhizomes of *Melpomene melanosticta* have been reported to maintain a sweet spicy fragance for tens of years (Smith and Moran, 1992); chemical analysis of this fragance should be of interest.

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Influence of Copper on Selected Physiological Responses in *Salvinia minima* and Its Potential Use in Copper Remediation

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ABSTRACT.—This study was designed to evaluate selected physiological responses of *Salvinia minima* to copper (Cu^{2+}) concentrations of 0.06 (control), 1.0, 2.0, 2.5, and 3.0 mg l^{-1}. The plants were grown under laboratory conditions of 25 ± 2°C, a light intensity of 120 µmol m^{-2} s^{-1}, and a 14-h photoperiod. After seven days of exposure to the Cu, *Salvinia* growth decreased gradually with an increase in Cu concentration resulting in a significant decline at 3.0 mg l^{-1}Cu. Similar results were obtained after 14 days of exposure. However, calculating growth using fresh weight showed a significant decline at 2.5 and 3.0 mg l^{-1}. After 14 days exposure, CO_2 assimilation decreased as the Cu concentration increased in the growth media. This decrease in CO_2 assimilation coincided with a similar decrease in photosynthetic pigments. Uptake of Cu significantly increased with the increase of Cu concentration in the growth media. This study demonstrated the potential of *Salvinia* to remediate Cu in concentrations 100 times what is currently found in freshwater environments.

Toxic heavy metal contamination is common in aquatic ecosystems due to both anthropogenic and natural sources. Runoff, industrial waste discharge and sewage effluent are the most frequent anthropogenic sources of aquatic contamination (Lee *et al.*, 1998).

Copper (Cu), an essential metal for plant growth is required in trace amounts (Guilizoni, 1991). Copper is a constituent of the chloroplast protein plastocyanin, which forms part of the electron transport chain linking the two photochemical systems of photosynthesis (Bowyer and Leegood, 1997). In addition, Cu functions as an activator or component of certain enzymes that are involved in a variety of biochemical processes, such as cytochrome *c* oxidase, and Cu-Zn superoxide dismutase (Linder, 1991).

Copper uptake appears to be a metabolically mediated process and there is evidence that Cu strongly inhibits the uptake of zinc (Zn) and *vice versa* (Hawf and Schmid, 1967). Generally, Cu toxicity causes chlorosis (Lewis, 1993; Vavilin *et al.*, 1995) and iron (Fe) deficiency by inhibiting translocation of Fe through the plant (Chaney, 1970; Lingle *et al.*, 1963; Wallace and DeKock, 1966). In addition, toxic levels of Cu inhibit root growth by damaging plasma membrane integrity (Marschner, 1995).

Copper is one of 13 metals listed as a priority pollutant by the U.S. Environmental Protection Agency (EPA) (Salomons *et al.*, 1995) and is among one of the most frequently discharged elements into the environment. It has been estimated that the global discharge of copper in aquatic systems is near 112×10^3 metric tons per year (Moore, 1991). Conventional remediation

methods such as precipitation, chemical oxidation or reduction, ion exchange, filtration, or evaporation processes are generally inefficient for removing metals in aquatic systems (Bervoets *et al.*, 1994). In contrast, the use of aquatic plants is currently under investigation as a viable alternative for remediation of a wide range of contaminants including heavy metals (Lee *et al.*, 1998). This cost-effective, plant-based approach to remediation takes advantage of the remarkable ability of plants to concentrate elements and compounds from the environment and to metabolize various molecules in their tissues (Salt *et al.*, 1998). Selecting plants as suitable candidates in phytoremediation must satisfy certain criteria such as reasonable tolerance to the contaminant in question, relatively high growth rate, and the ability to uptake and preferably metabolize the contaminant (Salt *et al.*, 1998).

The genus *Salvinia* (Salviniaceae) is comprised of one genus and 10 known species (Nauman, 1993). *Salvinia minima* Baker is a small, free-floating freshwater fern found in tropical and temperate regions of the world (DeBusk and Reddy, 1987) in areas such as North, South, and Central America, the West Indies, and Central America (Nauman, 1993). This plant can be found floating near the edges of slow moving streams and in nutrient enriched ponds. It is commonly referred to as water spangles and floating fern (Nauman, 1993). *Salvinia minima* demonstrated the ability to withstand aluminum (Al) concentrations of 20 mg l^{-1} through the manipulation of the media pH from 3.9 to near 7 within 24 hours (Gardner and Al-Hamdani, 1997). In addition, *Salvinia* showed considerable ability to accumulate cadmium (Cd II), 10,930 mg kg^{-1}; therefore it was suggested as a Cd II hyperaccumulater (Olguin *et al.*, 2002). *Salvinia* has the potential to double its population in approximately 3.5 days (Nichols *et al.*, 2000) making it a suitable candidate for phytoremediation.

This study was designed to evaluate the impact of Cu^{2+} concentrations of 0.06 (control), 1.0, 2.0, 2.5, and 3.0 mg l^{-1} on various physiological responses of *Salvinia* including plant growth, photosynthetic pigments, and CO_2 assimilation. The 0.06 mg l^{-1} concentration designated as the control was selected based on the average concentration of Cu in uncontaminated freshwater (Boyd, 1990). Copper uptake by *Salvinia*, grown at the different treatments, was determined.

Materials and Methods

The *Salvinia minima* utilized in this study was taken from stock material grown under greenhouse conditions for four years. Dr. David Whetstone, at the Jacksonville State University Herbarium, identified the plants, which were originally collected at a drainage ditch near Sanford, Florida (USA). Plants with a total of 15 fronds were placed into 60 (250 ml) Erlenmeyer flasks containing 125 ml of various Cu concentrations dissolved in 10% Hoagland solution with a pH of 6.5 (Hoagland and Arnon, 1938). Twelve flasks, samples, were used for each of the selected Cu concentrations, control (0.06); 1.0; 2.0; 2.5; and 3.0 mg l^{-1}. The initial fresh weight of the plants was recorded for each flask. The samples were placed randomly in the growth chamber and allowed to grow under conditions of 25 ± 2°C, a light intensity of 120 μmol m^{-2} s^{-1} and a 14-h

photoperiod. On day seven of the experiment, plant fresh weight and total frond number of six randomly selected flasks were assayed. In addition, 0.1 g fresh weight of tissue from each flask was used for chlorophyll *a* and *b*, and carotenoid determination. The remaining samples from these flasks were oven dried at 80°C for 48 hrs to be used later for Cu uptake determination. The media of the remaining six samples of each treatment were replaced with fresh solutions on day seven and the plants were allowed to grow for an additional seven days. On day 14 of the experiment, the same physiological parameters were determined as was CO_2 assimilation.

A separate experiment, as described above, was conducted with the exception that the media were not replaced at day seven. The existing media was filtered twice to reduce algal contamination. These plants were harvested on day 14 and oven dried at 80°C for 48 hrs to be used later for total Cu uptake. In addition, the medium of each sample was collected and the filters were analyzed for total Cu concentrations to insure a total accounting for Cu partitioning. The data obtained from this experiment was used to determine bioconcentration factors (BCF) and percent of Cu uptake.

Salvinia growth was expressed as doubling time (DT) in days. The doubling time was determined using the following equation: $DT = t \log 2 [\log (w_t w_o^{-1})]^{-1}$ (Moretti and Gigliano, 1988), where DT is the doubling time (days), t is the experiment duration (days), w_t is the final weight (or number of fronds), and w_o is the initial weight (or number of fronds).

Approximately 0.1 g fresh weight of each sample was used for measuring chlorophyll *a*, *b*, and carotenoid concentration. The plant was placed into 5 ml of N,N-Dimethylformamide (DMF) solution. The samples were incubated in the dark for 36 hrs at 4°C. Chlorophyll *a* and *b* was determined spectrophotometrically at wavelengths of 647 and 664.5 nm (Inskeep and Bloom, 1985). Carotenoid concentrations of the DMF extract were determined spectrophotometrically at a wavelength of 470 nm and the concentration was calculated using the formula of Doong et al. (1993).

Carbon dioxide assimilation and internal CO_2 concentrations of six randomly selected samples from each treatment were measured four hours after the onset of the light period on days seven and 14 of the treatments application. The selected plants of each sample were enclosed in a flow-through plexiglass assimilation chamber (4.5 by 11.8 by 7.3 cm) of a Li-Cor 6200 photosynthesis system (Lincoln, NE, USA) as described by McDermitt et al. (1989). Standard measurement conditions were 120 μmol m^{-2} s^{-1} photon flux density, 45 to 50% RH, and 25°.

Oven dried plant samples, ranging from 0.01-0.07 g, were digested according to procedures for Cu sampling outlined in the Buck Model 210 VGP Atomic Absorption Spectrophotometer Operating Manual (Buck Scientific, 1996). The samples were refluxed in 10 ml of 6N nitric acid for 15 min, just below the boiling point, and then 5 ml concentrated nitric acid (15.8N) was added. The reflux process was continued until the sample volume was reduced to approximately 5 ml. The samples were allowed to cool after which 2 ml H_2O and 5 ml 30% H_2O_2 were added to each sample. The samples were warmed

TABLE 1. Influence of different Cu concentrations on *Salvinia* growth. Plant growth is expressed as doubling time (DT) based on fresh weight and frond number for both seven and 14 days of exposure. LSD (P = 0.05) = Least Significant Difference value for difference between means within a column. Same letter denotes no statistical difference. Lower case letters denote differences between treatments within a day and upper case letters denote differences between days within treatments. N = 24

	DT based on mean frond number (days)		DT based on mean fresh weight (days)	
	Length of exposure (days)			
Cu (mg l^{-1})	7	14	7	14
0.06 (control)	7.61 aA	8.23 aA	5.70 aA	7.68 aB
1.0	8.37 abA	8.44 aA	6.03 aA	8.39 abB
2.0	8.10 abA	8.65 abA	6.83 aA	8.79 abB
2.5	8.34 abA	9.17 abA	7.87 abA	9.22 bB
3.0	9.84 bA	9.82 bA	8.92 bA	10.06 bB

slowly adding 1 ml of 30% H_2O_2 as needed until effervescence subsided. After cooling again, reflux of the samples was continued for 15 min. using HCl in aratio of 1 ml for each 2 ml of sample. After cooling, the samples were brought to 25 ml with distilled H_2O. Standards were established using Cu concentrations of 0.05, 0.1, 0.5, 1.0, 5.0, and 10.0 mg l^{-1} and absorbance was measured for each sample using the atomic absorption spectrophotometer.

Copper concentrations in the growth media were determined following the digesting procedure outlined in the Buck Model 210 VGP atomic absorption spectrophotometer-operating manual (Buck Scientific, 1996). To the sample media, 2 ml nitric acid (15.8N) and 5 ml HCl was added. The samples were refluxed until approximately one quarter of the media remained and the volume brought back to 125 ml with distilled H_2O. The Cu concentration of each sample was determined as described above. The BCF was determined as the ratio of Cu concentration in the plant tissue to the concentration in the external media (Spacie *et al.*, 1995).

The experiments were statistically analyzed as a randomized complete block design (Steel and Torrie, 1980). This design ensured that observed differences in plant performances were largely due to treatments rather than variation among the four blocks. The block in this study represent the replicate series of each experiment conducted at different times. Mean separations for the treatments with significant F values (P = 0.05) of ANOVA analysis were based on the least significant difference (LSD) test (Steel and Torrie, 1980).

RESULTS

After seven days of exposure to the various Cu concentrations, the only significant reduction in *Salvinia* growth was obtained on the medium containing 3.0 mg l^{-1} Cu (Table 1). Similar results were obtained at the end of day 14 of treatments exposure. Additionally, using fresh weight to calculate DT, the data showed that Cu concentrations of 2.5 mg l^{-1} significantly reduced

TABLE 2. Concentrations of chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids in salvinia as influenced by different Cu concentrations after seven and 14 days. LSD (P = 0.05) = Least Significant Difference value for difference between means within a column. Same letter denotes no statistical difference. Lower case letters denote differences between treatments within a day and upper case letters denote differences between days within treatments. N = 24

	Chl a (mg g^{-1} fr wt.)		Chl b (mg g^{-1} fr wt.)		Carotenoid (mg g^{-1} fr wt.)	
	Length of exposure (days)					
Cu conc (mg l^{-1})	7	14	7	14	7	14
0.06 (control)	11.67 aA	12.82 aB	6.93aA	7.01 aA	3.14aA	3.61 aA
1.0	6.02 bA	5.98 bA	3.82 bA	3.09 bB	0.47 bA	0.37 bA
2.0	5.10 bA	3.67cB	3.25 cA	1.86cB	0.30 bA	0.07 bA
2.5	5.60 bA	4.36 cB	3.66bcA	2.40 dB	0.42 bA	0.16bA
3.0	5.29 bA	4.57 cB	3.35 bcA	2.43 dB	0.33bA	0.17bA

Salvinia growth at the end of day14 of treatment (Table 1). *Salvinia* growth, measured as frond number DT, was not significantly different in the presence of 1.0, 2.0, 2.5 mg l^{-1} Cu from that of 3.0 mg l^{-1} Cu. Comparing the same treatments, day 14 data analysis was shown that significant reduction in growth at 3.0 mg l^{-1} Cu in contrast to that of 1.0 mg l^{-1} Cu. Slight deviation from these findings was obtained from data analysis using plant fresh weight DT that reveled growth was significantly less in 3.0 mg l^{-1} Cu than at 1.0 and 2.0 mg l^{-1} Cu. Using frond number DT, no differences were noted in *Salvinia* growth between day seven and 14 at each of the various Cu concentrations. However, there was a significant increase in fresh weight DT values, which corresponds to a significant reduction in growth, at the end of 14 days for all treatments in comparison to those obtained at day seven (Table 1).

After seven days exposure, the increase in Cu concentrations from 1.0 to 3.0 mg l^{-1} had similarly influenced chlorophyll *a* and *b* and carotenoid concentrations and significantly reduced these photosynthetic pigments, relative to the control. The only exception to this finding was Chlorophyll *b*, which was significantly higher at 1.0 than at 2.0 mg l^{-1} Cu (Table 2). In general, a reduction in *Salvinia* photosynthetic pigments obtained at day 14 reflected that seen at seven days (Table 2). However, at 1.0 mg l^{-1} Cu, chlorophyll *a* concentration was significantly higher than that of the other treatments except for the control, 0.06 mg l^{-1}. The gradual decrease in chlorophyll *b* was interrupted by an increase in Cu from 2.5 to 3.0 mg l^{-1} in comparison to 2.0 mg l^{-1}. Chlorophyll *a* accumulation in *Salvinia* significantly increased at day 14 in comparison to day seven within the control (Table 2). However, chlorophyll *b* and carotenoid concentrations were not significantly affected. In comparison the photosynthetic pigments between days seven and 14 for each treatment, carotenoid concentrations showed no significant difference whereas *Salvinia* grown at Cu concentrations of 1.0 mg l^{-1} and higher revealed a significant reduction in chlorophyll *a* and *b*, with the exception of chlorophyll *a* at 1.0 mg l^{-1}.

After fourteen days of Cu exposure there were significant decreases in CO_2 assimilation for all Cu concentrations from 1.0 to 3.0 mg l^{-1} as compared to the

TABLE 3. Carbon dioxide assimilation and internal CO_2 in *Salvinia* after 14 days of varying Cu exposure. LSD (P = 0.05) = Least Significant Difference value for difference between means within a column. Same letter denotes no statistical difference. Lower case letters denote differences between treatments within a day and upper case letters denote differences between days within treatments. N = 12.

Cu conc (mg l^{-1})	CO_2 assimilation (umol $m^{-2}s^{1}$)	Internal CO_2 (ul l^{1})
0.06 (control)	2.32 a	351.37 a
1.0	1.51 b	351.77 a
2.0	1.12 c	350.92 a
2.5	1.10 c	350.92 a
3.0	1.01 c	350.06 a

control (Table 3). However, treatments receiving 2.0, 2.5, and 3.0 mg l^{-1} did not differ. Furthermore, there was a 25.8, 27.1, and 33.1% increase in CO_2 assimilation in plants grown at 1.0 mg l^{-1} Cu when compared to that obtained in plants receiving higher Cu concentrations. Internal CO_2 concentrations were not different among plants grown in all treatments (Table 3).

After seven days of growth, Cu accumulation was significantly higher in plants receiving 1.0 to 3.0 mg l^{-1} Cu in comparison to the control (Table 4). Furthermore, Cu accumulation in *Salvinia* grown at 1.0 mg l^{-1} was significantly lower than those plants receiving higher concentrations. At the end of 14 days, *Salvinia* still showed an increase of Cu uptake correlated with increasing Cu concentrations in the growth media. However, examining the individual treatments showed that at 1.0 mg l^{1} Cu, *Salvinia* accumulation was similar to that of the 0.06 and 2.0 mg l^{-1} concentration. Copper uptake of the media containing 3.0 mg l^{-1} was the highest in comparison to the other treatments with a 43.9% increase in comparison to the nearest treatment, 2.5 mg l^{-1}. Whereas, Cu accumulation in *Salvinia* was similar in those plants grown at 2.0 and 2.5 mg l^{-1}. With the exception of those plants grown at 0.06 and 3.0 mg l^{-1} Cu, *Salvinia* uptake of Cu was significantly higher during the first seven days in comparison to 14 days of the experiment (Table 4). These results coincide with the BCF calculation of each treatment after 14 days of Cu exposure that was nearly 20-fold higher in plants at 3.0 mg l^{-1} Cu in comparison to the control and twice as high in plants grown in 2.5 mg l^{-1} Cu (Table 4).

DISCUSSION

The association between reduced growth and increased Cu concentration (Table 1) has also been observed in several other aquatic plants such as *Lemna minor* L. (Teisseire *et al.*, 1998), *Potamogeton pectinatus* L., *Vallisneria spiralis* L., *Hydrilla verticillata* (L.f.) Royle (Guilizzoni 1991), and *Elodea nuttallii* (Planch.) St. John (Van der Werff and Pruyt, 1982). Sarkar and Jana (1986) reported that *Azolla pinnata* R. Br. growth was significantly reduced following exposure to 2.0 mg l^{-1} Cu, whereas plants at 1.0 mg l^{-1} exhibited growth similar

TABLE 4. Cu uptake (μg Cu g^{-1} d. wt) in *Salvinia* after seven and 14 days of exposure to varying concentrations. LSD (P = 0.05) = Least Significant Difference value for difference between means within a column. Same letter denotes no statistical difference. Lower case letters denote differences between treatments within a day and upper case letters denote differences between days within treatments. N = 12. Bioconcenration factor (BCF) was determined as the ratio of Cu concentration in the plant tissue relative to the concentration in the external media after 14 days of exposure. N = 6.

	Length of exposure (days)		
Cu (mg l^{-1})	7	14	BCF
0.06 (control)	1.50.35 aA	167.33 aA	308.91 a
1.0	1833.54 bA	735.62 abA	1390.08 ab
2.0	2934.90 cA	1545.49 bcB	2132.87 bc
2.5	3111.67 cA	2413.75 cB	3111.63 c
3.0	3519.20 cA	4304.44 dB	4304.44 d

to the control after 28 days of incubation. A possible explanation of decreasing *Salvinia* growth with increasing Cu concentrations might be attributed to Cu-induced ethylene production. Matoo *et al.*, (1986) reported that intercellular membrane and organelle disintegration in giant duckweed (*Spirodela oligorrhiza* (Kurz) Hegelm) resulted from induced ethylene production by copper. In our study, reduction in *Salvinia* growth was directly related to a decline in CO_2 assimilation as a function of Cu increase (Table 3). The negative impact of increasing Cu concentration on CO_2 assimilation was reported to be due to the inhibitory effect on the electron transport of photosystem II (PS II) (Sarkar and Jana, 1986; Renganathan and Bose, 1989). The decline in PS II was attributed to degradation and leakage of the chloroplast membrane induced by Cu (Ouzounidou *et al.*, 1993). Furthermore, the decline in CO_2 assimilation might be influenced by the reduction in photosynthetic pigments, which was associated with increasing Cu concentrations (Table 2). A reduction in photosynthetic pigment concentration in *Salvinia* has been associated with an increase in metal contamination (Gardner and Al-Hamdani, 1997; Nichols *et al.*, 2000). Sarkar and Jana (1986) attributed the reduction in chlorophyll concentration to the influence of Cu on declining chlorophyllase activity. Furthermore, the decline in CO_2 assimilation and photosynthetic pigment might be related to membrane destruction by lipid peroxidation, which was found to be associated with an increase in Cu accumulation (Halliwell and Gutteridge 1984). Mattoo *et al.* (1986) reported that free radical formation was induced by an increase in Cu concentration that later reformed into H_2O_2. As a defense mechanism against increasing free radicals, plants usually respond by increasing catalase activity (Foyer *et al.*, 1994). Catalase activity was found to decline gradually in duckweed as Cu concentrations increased above 0.2 mg l^{-1} in the nutrient media (Teisseire *et al.*, 1998).

Copper uptake by *Salvinia* was significantly higher with an increase in Cu concentration in the growth media (Table 4). With the exception of those plants grown in 3.0 mg l^{-1} Cu, the concentration of Cu in *Salvinia* was significantly higher at the end of seven days of plant growth in comparison to that at day 14.

In calculating Cu uptake as μg g^{-1} plant dry weight, growth should be considered a factor in interpreting the values at each individual treatment. This conclusion equally reflects Cu uptake in plants grown in 3.0 mg l^{-1} Cu, where growth was severely affected at the end of seven days with a chlorotic and necrotic appearance that advanced with time. However, the BCF was highest for the plants grown in 3.0 mg l^{-1} Cu followed by a decreasing order as the Cu concentration decreased in the media. In comparison with other species, *Salvinia* uptake of Cu was comparable to that obtained with monkey flower (*Mimulus guttatus* DC.; Tilstone and MacNair, 1997) and iceplant (*Mesembryanthemum crystallinum* L.; Thomas *et al.*, 1998) using equivalent Cu concentrations and incubation periods. In addition, *Salvinia* has the ability to survive and grow under highly eutrophic environments unsuitable for other species found in similar environments such as *Azolla* and duckweed (Reddy and DeBusk, 1985). This can be used as an additional indication that *Salvinia* can be considered an essential agent in phytoremediation.

In conclusion, this study demonstrated that increases in Cu concentration from 1.0 to 3.0 mg l^{-1} negatively impacted plant growth, photosynthetic pigments, and CO_2 assimilation. However, the reduction in plant growth was not severe enough to totally inhibit plant growth even at Cu concentrations of 3.0 mg l^{1}. *Salvinia* demonstrated the ability to accumulate significant concentrations of Cu in its tissues. This, in addition to its high growth rate and ease in harvesting, make it a possible candidate for phytoremediation. However, further research should be implemented to investigate the performance of *Salvinia* under field conditions.

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Bark Spore Bank of Ferns in a Gallery Forest of the Ecological Station of Panga, Uberlândia–MG, Brazil

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ABSTRACT.—Information about fern spore banks is restricted to the soil systems. As the dispersion of spores occurs by means of air, it is possible to have viable spores on tree bark. Thus, it is important to know if on this kind of substrate, which is thinner and apparently more susceptible to desiccation than the soil, the spores can survive for any length of time, forming transient or persistent spore banks. Samples of bark were collected from ten angiosperm trees in March 1997 and from fifteen trees in February and September 1998. The samples collected in March 1997 contained from 0.05 to 7.19 gametophytes cm ^{2} of cultured bark, those of February 1998 from 0.11 to 4.22 gametophytes cm ^{2}, and in September 1998 from 0.32 to 5.0 gametophytes per square cm. Although the cerrado region is characterized by climatic seasonality, this seasonality was not observed in relation to number of viable spores on barks. As a consequence of the casual spore dispersion pattern, the bark spore bank has a random distribution among the trunks. Ten species were identified on barks collected in February 1998 and fifteen in September 1998, one of them epiphytic (*Phlebodium areolatum* (Humb. & Bonpl. ex Willd.) J. Sm.) and the others terrestrial species. *Thelypteris* was the most frequent genus in the analyzed samples. The results obtained show the potential for these substrates to retain viable spores that can participate in the regeneration process and population dynamics of the pteridophyte flora. Moreover, the existence of viable spores of terrestrial species on tree bark does not answer an important question—why do terrestrial species not establish themselves on trees?

Information about fern spore banks is restricted to the soil (Carroll and Ashton, 1965; Wee, 1974; Strickler and Edgerton, 1976; Pérez-García *et al.*, 1982; During and ter Horst, 1983; During *et al.*, 1987; Leck and Simpson, 1987; Hamilton, 1988; Lindsay and Dyer, 1990; Milberg, 1991; Dyer and Lindsay, 1992; Milberg and Anderson, 1994; Penrod and McCormick, 1996; Raffaele, 1996; Schneller and Holderegger, 1996; Simabukuro *et al.*, 1998, 1999; Ranal, 2003). As the dispersion of spores occurs by means of the air, it is possible that viable spores present on the bark of trees could germinate under appropriate conditions. Thus, it is important to know if on this kind of substrate, which is thinner and apparently more susceptible to desiccation than soil, the spores can survive long enough to form a transient or persistent bank. These spores can participate in the population dynamics, particularly of epiphyte species. The role of these fern spores could be amplified after the fall of trees, if spores of terrestrial species could survive in this kind of substrate. In vertical position the spore reception is maximized on the trunks. On the other hand, in horizontal positions (dead trees), wind action and self-defense of trees decreases. As a consequence, water retention and decomposition activities increase, making the germination process on this new substrate easier. In this sense, it will be possible to consider that this bark spore bank has the same role recognized for soil spore bank, that is, this bank could take an important role in

propagation and in preservation of fern species as pointed out for soil spore bank by Lindsay *et al.* (1992), Dyer (1994), and Dyer and Lindsay (1996); in sexual process and in genetic variability (Milberg, 1991); and in regeneration process of forest gaps.

In this context, the aim of this study was to investigate the existence of a bark spore bank on trunks of angiosperm trees in the gallery forest of the Ecological Station of Panga, Uberlândia–MG, Brazil. This being the case, the purpose was to characterize this bank in relation to quantity of viable spores and fern species composition.

Materials and Methods

Bark was collected from ten angiosperm trees in March 1997, and from fifteen in February and September 1998. These trees are growing in the gallery forest of the Ecological Station of Panga, Uberlândia, Minas Gerais, Brazil, situated at 19°09′20″–19°11′10″ S, 48°23′20″–48°24′35″ W, at an elevation of approximately 800 m. This Station has 409.5 ha occupied by cerrado *sensu lato* (Schiavini and Araújo, 1989; Ratter, 1992). The region is characterized by an Aw climatic pattern (Köppen, 1948) with a wet and hot season from October to March and a dry and cold season from April to September (Ranal, 2003). Random sampling was used to mark the trees for the bark collection. All trees were adult individuals, with more than 15 cm in circumference at 1.30 m height and with sufficient bark to extract by scratching with a knife, without reaching live tissues. It means that dead bark was extracted (outer bark or rhytidome according to Esau, 1977), a perennial rhytidome with slow detachment. Bark samples were collected at about 1.30 m height around the trunks. As in 1998 the two collections were done on the same trees, the bark extraction was done at about 1.30 m height in February and at about 1.50 m in September.

After collection, each bark sample was placed in a plastic bag which was labelled and immediately closed to prevent contamination. In the laboratory bark samples were manually mixed and converted into small pieces and powder, inside the bags. Each bark sample was divided into sub-samples and spread over sterile sand in quadrangular, transparent, covered plastic boxes of 50 cm^3 (experimental units), moistened with nystatin suspension (10,000 units nystatin per mL in DPBS—Dulbecco's Phosphate Buffered Saline; 1 mL per 100 mL of distilled water) and later, if necessary, with distilled water. Near the end of the experiments, when young sporophytes presented the first signals of chlorosis, the cultures were moistened with nutrient solution (Meyer *et al.*, 1963). Each experimental unit received 15 g of sterile sand and 1 g of bark. This quantity of bark formed a layer of approximately 5 mm thickness. Samples were maintained at 20.9–23.2°C, 35.77 ± 6.71 μmol m^{-2} s^{-1} PAR (mean ± standard deviation) for the March 1997 collection, at 21.4–24.4°C, 35.77 ± 6.71 μmol m^{-2} s^{-1} PAR for the February 1998 collection, and at 21.8–22.9°C, 30.84 ± 6.09 μmol m^{-2} s^{-1} PAR for the September 1998 collection. All samples were subjected to continuous white fluorescent light. Radiation measurements were made using a LI-COR LI-250 light meter and a LI-190SA quantum sensor.

Sterilized soil samples (10 replicates) were maintained under the same manipulation conditions to assess the level of contamination by foreign spores. The superficial area of cultured barks was used to calculate the number of gametophytes and sporophytes formed per square centimeter. As bryophytes were the first colonizer of the barks, fern gametophytes were counted 2–3 months after each collection, when they reached adult form, becoming easily visible.

The number of gametophytes formed was the criterion used to evaluate viable spores on the bark samples. Sporophytes were counted when cultures were two (February collection) or three months old (March and September collections). The criterion to count sporophytes was the presence of a perceptible crozier when viewed under stereomicroscope. At the end of the experiments young sporophytes were transplanted to bags containing soil and were maintained under greenhouse conditions until the production of fertile leaves when they were collected. The sporophytes collected were prepared and deposited at HUFU and SP.

The experimental units were randomly distributed in laboratory conditions, with two sub-samples per tree for March 1997 collection (20 cells) and four subsamples per tree for February and September 1998 collection (60 cells for each collection date). The number of gametophytes and sporophytes formed per square centimeter of cultured bark, as well as the percentage of gametophytes forming sporophytes were submitted to the Shapiro-Wilk (normality of populations) and Bartlett or Cochran tests (homogeneity between variances). If the original data exhibited normality and homogeneity, they were submitted to analysis of variance and Tukey test. If the original and transformed data showed non-normality and/or heteroscedasticity, non-parametric statistical tests were used (Kruskal-Wallis and Wilcoxon-Mann-Whitney tests). Comparisons between the bark spore bank of the wet and dry seasons were carried out using the Mann-Whitney test. Pearson correlations were made to detect associations between number of gametophytes formed on cultured barks and tree characteristics (height, circumference, N, P, K, Ca, Mg, S, Fe, B, Cu, Mn, and Zn content).

Bark samples collected were chemically analyzed in the Laboratory of Leaf Analysis of the Federal University of Uberlândia, according to Miyazawa *et al.* (1999).

Results

All analyzed trees presented viable spores in their outer bark (Tables 1–3). The size of the bark spore bank, evaluated in relation to gametophytes formed, varied from 0.05 to 7.19 gametophytes cm^{-2} of cultured bark. There is no seasonality in the bark spore bank (Table 4). Half of the analyzed trees showed no differences between February (wet season) and September (dry season) collections; three showed increase in number of viable spores and four showed decrease in number of viable spores at the end of the dry season. There is no association between angiosperm species, tree size or chemical composition of the bark and the number of viable spores on barks (Tables 1–3, 5, 6). The same

TABLE 1. Gametophytes and sporophytes formed per square centimeter of cultured barks of angiosperms occurring in a gallery forest of Ecological Station of Panga, Uberlândia–MG, Brazil and their reproductive success measured by percentage of gametophytes forming sporophytes (mean ± standard deviation). March 1997 collection.

Angiosperm Species	Family	Gametophytes cm^{-2}	Sporophytes cm^{-2}	% sporophytes
Psidium rufum Mart. ex DC.[1]	Myrtaceae	7.19 ± 0.49 a	2.68 ± 0.38 a	37.24 ± 2.76 ab
tree in decomposition	—	2.15 ± 0.30 b	0.82 ± 0.43 b	39.82 ± 25.56 ab
Luehea divaricata Mart.	Tiliaceae	1.93 ± 0.41 bc	0.37 ± 0.23 b	18.16 ± 8.00 ab
tree in decomposition	—	1.56 ± 0.47 bcd	0.33 ± 0.20 b	20.19 ± 6.80 ab
Unidentified sp.[1]	—	0.89 ± 0.87 bcd	0.00 ± 0.00 b	0.00 ± 0.00 b
Cupania vernalis Cambess.	Sapindaceae	0.59 ± 0.22 bcd	0.00 ± 0.00 b	0.00 ± 0.00 b
Chrysophyllum marginatum Radlk.	Sapotaceae	0.48 ± 0.005 cd	0.05 ± 0.07 b	10.00 ± 14.14 b
undentified sp.[1]	—	0.49 ± 0.39 cd	0.29 ± 0.26 b	56.25 ± 8.84 a
Matayba guianensis Aubl.	Sapindaceae	0.09 ± 0.0 d	0.00 ± 0.00 b	0.00 ± 0.00 b
Terminalia brasiliensis Eichl.	Combretaceae	0.05 ± 0.07 d	0.00 ± 0.00 b	0.00 ± 0.00 b
W		**0.9813**	**0.9433**	**0.9301**
Cochran		**0.4473**	**0.3718**	**0.6227**
$F_{9;\ 10}$		53.29**	27.55**	7.78**

W: Shapiro-Wilk test ($\alpha = 0.05$); boldfaced values indicate normality of populations ($P > 0.05$). Boldface values for Cochran test indicate homogeneity between variances. *F*: value of Snedecor's distribution, including the degrees of freedom; ** $P \leq 0.01$. Means followed by the same letter in each column are not significantly different based on the Tukey test ($\alpha = 0.05$). [1] Tree with *Microgramma persicariifolia*.

angiosperm species presented high or low number of viable spores, trees with different dimensions presented similar number of viable spores without any significant correlation between tree size and bark bank size, and weak tendency related to chemical composition could be observed. Moderate to substantial negative correlations (Table 6), according to the criterion adopted by Miller (1994), were detected for nitrogen (February collection), magnesium, and copper content (September collection).

Sporophyte production ranged from zero to 2.68 sporophytes cm^{-2} of cultured bark and the reproductive success (percentage of gametophytes forming sporophytes) from zero to 62.10 % (Tables 1–3).

Host trees of viable fern spores are presented on table 7. Ten fern species were recognized in barks collected in February 1998 and 15 species in barks collected in September 1998. Each analyzed tree presented from two to ten fern species in their barks. *Pityrogramma calomelanos* (L.) Link var. *calomelanos* and *Thelypteris opposita* (Vahl) Ching were broadly distributed, occurring in 13 trees, from the 25 analyzed; *Phlebodium areolatum* (Humb. & Bonpl. *ex* Willd.) J. Sm., *Pteris vittata* L., *Thelypteris burkartii* Abbiatti, and *T. mosenii* (C. Chr.) C.F. Reed appeared only in one tree. *Phlebodium areolatum* is epiphyte and the others are terrestrial species. *Microgramma persicariifolia* (Schrad.) Presl was found growing as an epiphyte in the forest, but no

TABLE 2. Gametophytes and sporophytes formed per square centimeter of cultured barks of angiosperms occurring in a gallery forest of Ecological Station of Panga, Uberlândia–MG, Brazil and their reproductive success measured by percentage of gametophytes forming sporophytes (mean ± standard deviation). February 1998 collection.

Angiosperm Species	Family	Gametophytes cm^{-2}	Sporophytes $cm^{-2(1)}$	% sporophytes
Copaifera langsdorffii Desf.[2]	Caesalpiniaceae	4.22 ± 0.42 a	2.29 ± 0.30 a	54.22 ± 2.51 a
Eugenia ligustrina Miq.	Myrtaceae	2.80 ± 0.42 ab	1.22 ± 0.23 b	43.78 ± 6.54 ab
Endlicheria paniculata (Spreng.) Macbride[3]	Lauraceae	2.68 ± 0.99 ab	1.59 ± 0.70 ab	58.67 ± 10.36 a
Eugenia ligustrina	Myrtaceae	2.26 ± 0.54 abc	0.91 ± 0.32 bc	40.86 ± 12.94 ab
Aspidosperma cylindrocarpum Muell. Arg.	Apocynaceae	2.06 ± 0.36 abc	0.51 ± 0.14 cd	24.70 ± 4.41 ab
Tapirira guianensis Aubl.	Anacardiaceae	1.94 ± 0.54 abc	0.91 ± 0.20 bc	49.07 ± 15.61 ab
Coussarea hydrangeae-folia Benth. & Hook. f.	Rubiaceae	1.92 ± 0.59 abc	0.32 ± 0.14 def	16.76 ± 6.59 ab
Eugenia florida DC.	Myrtaceae	1.55 ± 0.57 abc	0.28 ± 0.11 def	19.64 ± 8.27 ab
Inga affinis DC.	Mimosaceae	1.49 ± 0.30 abc	0.45 ± 0.16 cde	30.27 ± 6.32 ab
Luehea divaricata Mart.	Tiliaceae	1.32 ± 0.22 abc	0.56 ± 0.09 cd	43.14 ± 7.94 ab
dead tree	—	0.83 ± 0.39 bc	0.02 ± 0.04 f	1.56 ± 3.12 b
Tapirira guianensis	Anacardiaceae	0.75 ± 0.29 bc	0.32 ± 0.22 def	44.17 ± 24.10 ab
Aspidosperma cylindrocarpum	Apocynaceae	0.50 ± 0.08 bc	0.15 ± 0.11 def	29.64 ± 21.61 ab
Tapirira guianensis	Anacardiaceae	0.17 ± 0.10 c	0.02 ± 0.04 f	25.00 ± 50.0 ab
Luehea divaricata	Tiliaceae	0.11 ± 0.11 c	0.06 ± 0.08 ef	41.67 ± 50.0 ab
W		0.9435	**0.9728**	0.8774
Bartlett		—	**24.1624**	—
$F_{14;\ 45}$		—	31.42**	—
H		52.11**	—	30.32**

W: Shapiro-Wilk test ($\alpha = 0.05$); boldfaced value indicates normality of populations ($P > 0.05$). Boldface value for Bartlett test indicates homogeneity between variances. *F*: value of Snedecor's distribution, including the degrees of freedom; ** $P \leq 0.01$. *H*: Kruskal-Wallis test; ** $P \leq 0.01$. Means followed by the same letter in each column are not significantly different based on the Wilcoxon-Mann-Whitney or Tukey test ($\alpha = 0.05$). [1] Data submitted to square root plus 0.5 transformation for the adjustment to normality and homogeneity; means and standard deviation are original numbers; numbers and letters related to statistics are based on transformed data. [2] Tree with *Microgramma persicariifolia*. [3] Bark collected of the horizontal part of the stem.

gametophyte of this species was found in the analyzed barks. *Thelypteris* was the most frequent genus among the analyzed samples (Table 8).

DISCUSSION

The bark spore bank of the analyzed trees is smaller than the soil spore bank of the middle and edge of the same gallery forest, at the first centimeters of the soil column (2–7 cm depth), in the wet season, and similar to that soil spore bank in deeper soil column (15–22 cm depth). Soil samples of the gallery forest of the Ecological Station of Panga could reach 29.52 gametophytes cm^{-2} of

TABLE 3. Gametophytes and sporophytes formed per square centimeter of cultured barks of angiosperms occurring in a gallery forest of Ecological Station of Panga, Uberlândia–MG, Brazil and their reproductive success measured by percentage of gametophytes forming sporophytes (mean ± standard deviation). September 1998 collection.

Angiosperm Species	Family	Gametophytes cm^{-2}	Sporophytes cm^{-2}	% sporophytes
Copaifera langsdorffii Desf.[1]	Caesalpiniaceae	2.52 ± 0.32 ab	1.18 ± 0.22 b	46.81 ± 8.80 a
Eugenia ligustrina Miq.	Myrtaceae	0.83 ± 0.36 bc	0.23 ± 0.10 d	29.38 ± 15.60 a
Endlicheria paniculata (Spreng.) Macbride[2]	Lauraceae	5.00 ± 0.73 a	1.86 ± 0.53 a	37.12 ± 7.82 a
Eugenia ligustrina	Myrtaceae	0.73 ± 0.39 bc	0.26 ± 0.19 d	35.02 ± 13.54 a
Aspidosperma cylindrocarpum Muell. Arg.	Apocynaceae	1.54 ± 0.35 abc	0.45 ± 0.26 cd	30.15 ± 17.84 a
Tapirira guianensis Aubl.	Anacardiaceae	1.75 ± 0.50 abc	1.02 ± 0.23 bc	62.10 ± 25.65 a
Coussarea hydrangeaefolia Benth. & Hook. f.	Rubiaceae	0.93 ± 0.28 bc	0.11 ± 0.09 d	14.45 ± 14.08 a
Eugenia florida DC.	Myrtaceae	1.30 ± 0.05 abc	0.70 ± 0.28 bcd	54.06 ± 21.82 a
Inga affinis DC.	Mimosaceae	1.95 ± 0.43 abc	0.24 ± 0.15 d	14.09 ± 11.86 a
Luehea divaricata Mart.	Tiliaceae	2.47 ± 0.49 ab	1.10 ± 0.56 bc	46.54 ± 25.60 a
dead tree	—	2.28 ± 1.45 abc	0.70 ± 0.24 bcd	34.78 ± 13.26 a
Tapirira guianensis	Anacardiaceae	0.88 ± 0.12 bc	0.52 ± 0.24 bcd	58.61 ± 20.19 a
Aspidosperma cylindrocarpum	Apocynaceae	1.84 ± 0.36 abc	1.00 ± 0.13 bc	56.75 ± 15.61 a
Tapirira guianensis	Anacardiaceae	0.43 ± 0.29 c	0.13 ± 0.11 d	30.42 ± 27.50 a
Luehea divaricata	Tiliaceae	0.32 ± 0.19 c	0.17 ± 0.12 d	47.5 ± 41.13 a
W		0.9078	**0.9708**	**0.9837**
Bartlett		—	**23.9762**	**14.8281**
$F_{14;\ 45}$		—	14.1975**	2.1431*
H		51.6850**	—	—

W: Shapiro-Wilk test ($\alpha = 0.05$); boldfaced values indicate normality of populations ($P > 0.05$).

cultured soil, in the wet season, when collected at 2–4 cm depth, and ranged from 0.13 to 6.84 gametophytes cm^{-2} from 15 to 22 cm depth (Ranal, 2003). Dyer and Lindsay (1992) registered more than 30 gametophytes cm^{2} from surface to 2.5 cm depth of soil collected in North Carolina and 0.46 gametophytes cm^{-2} at 20.0–22.5 cm.

Similar results in relation to reproductive success were obtained for soil spore bank studies (0.76 to 63.33 % gametophytes producing sporophytes) of the same gallery forest (Ranal, 2003).

Periodic observations indicate that for some species of the Ecological Station of Panga, production of new leaves occurs in October–November, at the beginning of the rainy season, and the production of fertile leaves occurs in

TABLE 4. Simple comparisons for gametophytes formed in bark samples collected in February and September 1998, in the gallery forest, Ecological Station of Panga, Uberlândia–MG. The mean values and the dispersion measurements are included on tables 2 and 3.

Angiosperm Species	*U* value	P value
Copaifera langsdorffii Desf.	16.0	0.0286
Eugenia ligustrina Miq.	16.0	0.0286
Endlicheria paniculata (Spreng.) Macbride	16.0	0.0286
Eugenia ligustrina	16.0	0.0286
Aspidosperma cylindrocarpum Muell. Arg.	14.0	0.1140
Tapirira guianensis Aubl.	8.5	0.8860
Coussarea hydrangeaefolia Benth. & Hook. f.	16.0	0.0286
Eugenia florida DC.	8.0	1.0000
Inga affinis DC.	13.0	0.2000
Luehea divaricata Mart.	16.0	0.0286
dead tree	15.0	0.0571
Tapirira guianensis	11.0	0.4860
Aspidosperma cylindrocarpum	16.0	0.0286
Tapirira guianensis	14.0	0.1140
Luehea divaricata	12.0	0.3430

U: statistic of the Mann-Whitney test. P: probability to accept or reject the null hypothesis. $P > 0.05$ means that the two medians are not significantly different. $P < 0.05$ means that the two medians are significantly different.

December–January. Spore dispersal occurs from December for precocious leaves to March–April for late leaves, depending on the annual rainfall distribution. This seasonality in spore production was not observed in the bark spore bank, but was detected for soil spore bank in the first centimeters of soil

TABLE 5. Bark chemical composition of angiosperm species occurring in the gallery forest of Ecological Station of Panga, Uberlândia–MG, Brazil.

	$g\ Kg^{-1}$						$mg\ Kg^{-1}$				
Angiosperm Species	N	P	K	Ca	Mg	S	Fe	B	Cu	Mn	Zn
Aspidosperma cylindrocarpum Muell. Arg.	17.2	1.2	1.5	8.6	1.2	1.9	17508.0	15.0	18.0	252.0	23.0
Aspidosperma cylindrocarpum	19.0	1.5	1.5	10.3	1.3	2.0	15030.0	26.0	17.0	203.0	21.0
Copaifera langsdorffii Desf.	11.4	0.9	1.5	4.5	1.0	1.3	11503.0	23.0	13.0	350.0	20.0
Coussarea hydrangeaefolia Benth. & Hook. f.	13.9	0.9	0.5	7.7	0.8	1.1	6015.0	17.0	10.0	703.0	15.0
Endlicheria paniculata (Spreng.) Macbride	15.4	1.5	2.0	6.8	1.1	2.1	27000.0	17.0	25.0	1051.0	61.0
Eugenia florida DC.	12.1	0.8	1.5	26.8	1.2	1.4	16005.0	21.0	11.0	293.0	42.0
Eugenia ligustrina Miq.	13.5	0.7	1.0	24.9	1.6	1.3	7012.0	18.0	12.0	125.0	22.0
Eugenia ligustrina	12.1	0.7	1.0	20.8	1.2	1.0	6511.0	13.0	10.0	133.0	22.0
Inga affinis DC.	16.1	1.7	7.0	10.6	1.7	2.1	27000.0	21.0	25.0	675.0	40.0
Luehea divaricata Mart.	13.9	0.8	1.0	48.3	3.6	1.4	10000.0	11.0	17.0	1105.0	240.0
Luehea divaricata	15.7	0.9	1.0	43.3	2.3	0.9	16550.0	18.0	25.0	551.0	115.0
Tapirira guianensis Aubl.	13.2	0.8	1.0	43.9	1.9	1.0	5750.0	28.0	15.0	425.0	20.0
Tapirira guianensis	15.4	1.2	1.5	25.2	1.9	2.0	2250.0	23.0	22.0	675.0	55.0
Tapirira guianensis	13.9	0.9	1.0	26.8	2.6	1.0	5020.0	18.0	15.0	148.0	23.0
dead tree	11.7	0.8	3.0	19.3	1.1	0.9	7750.0	22.0	8.0	177.0	47.0

TABLE 6. Coefficients of the linear correlation (*r*) among tree characteristics and gametophytes per square centimeter of cultured bark collected in February and September 1998 in the gallery forest of the Ecological Station of Panga, Uberlândia–MG, Brazil.

Tree characteristics	*r* values[1]	P values	*r* values[2]	P values
Height (m)	−0.1014	0.3596	0.0932	0.3706
Circumference (cm)	0.1580	0.2869	0.0728	0.3983
N	−0.5003	0.0288	−0.3538	0.0979
P	−0.1228	0.3314	−0.1615	0.2827
K	0.3403	0.1073	0.0634	0.4112
Ca	−0.2815	0.1548	−0.0691	0.4033
Mg	−0.3348	0.1113	−0.4716	0.0380
S	−0.0176	0.4752	−0.0736	0.3971
Fe	0.1775	0.2634	0.0889	0.3763
B	−0.0678	0.4051	0.2245	0.2106
Cu	−0.3978	0.0710	−0.4591	0.0426
Mn	0.0298	0.4581	−0.2133	0.2227
Zn	0.0817	0.3861	−0.2651	0.1698

[1] linear correlation for gametophytes per square centimeter of cultured bark collected in February 1998. [2] linear correlation for gametophytes per square centimeter of cultured bark collected in September 1998. $P > 0.05$ means that null hypothesis was accepted and *r* was not considered as significantly different from zero by "Student's" *t* test. $P < 0.05$ means that null hypothesis was rejected and *r* was considered as significantly different from zero by "Student's" *t* test.

column, with high quantity of viable spores occurring at the end of the wet season and low quantity at the end of the dry season (Ranal, 2003).

Although *Copaifera langsdorffii* Desf. contains alkaloids and terpenoids in its bark (Souza and Silva, 2001), these substances apparently did not influenced the spore germination and gametophyte development of *Pityrogramma calomelanos* var. *calomelanos*, *Pteridium aquilinum* (L.) Kuhn var. *arachnoideum* (Kaulf.) Brade, *Thelypteris burkartii*, *T. conspersa* (Schrad.) A. R. Sm., *T. dentata* (Forssk.) E. St. John, *T. hispidula* (Decne) C. F. Reed, *T. opposita* (Vahl) Ching, and *T. patens* (Sw.) Small because viable spores of these species were maintained on its bark, with normal development until sporophyte production. Alkaloids and terpenoids are related to allelopathic mechanisms that inhibit the germination process (Inderjit and Dakshini, 1995).

Five of the species of the bark spore bank did not occur in soil samples of the gallery forest of the Panga Stream (*Phlebodium areolatum*, *Pityrogramma trifoliata* (L.) R.M. Tryon, *Pteridium aquilinum* var. *arachnoideum*, *Pteris vittata*, and *Thelypteris burkartii*). On the other hand, three of the 13 species registered by Ranal (2003) in the soil of the gallery forest of Panga did not occur in bark cultures (*Lygodium venustum* Sw., *Blechnum brasiliense* Desv., and *Blechnum occidentale* L.). Thus, there were 10 common species in the soil and bark spore bank.

The interpretation of these data is limited because these are the first results about bark spore banks and few angiosperm species were analyzed. Moreover, there is no assurance that all fern spores present on the barks could germinate and form sporophytes under the experimental conditions used in this study. Several environmental factors such as wind currents, rainfall, gravity, and

TABLE 7. Species that are able to form bark spore bank in the gallery forest of the Ecological Station of Panga, Uberlândia, MG.

Fern species	Host tree
Macrothelypteris torresiana (Gaud.) Ching	*Coussarea hydrangeaefolia* Benth. & Hook. f. dead tree *Eugenia florida* DC. *Inga affinis* DC. *Luehea divaricata* Mart. *Tapirira guianensis* Aubl.
Phlebodium areolatum (Humb. & Bonpl. *ex* Willd.) J. Sm.	*Tapirira guianensis*
Pityrogramma calomelanos (L.) Link var. *calomelanos*	*Aspidosperma cylindrocarpum* Muell. Arg. *Copaifera langsdorffii* Desf. *Coussarea hydrangeaefolia* dead tree *Endlicheria paniculata* (Spreng.) Macbride *Eugenia florida* *Eugenia ligustrina* Miq. *Inga affinis* *Luehea divaricata* *Tapirira guianensis*
Pityrogramma trifoliata (L.) R. M. Tryon	*Aspidosperma cylindrocarpum* dead tree *Endlicheria paniculata* *Eugenia ligustrina* *Inga affinis* *Luehea divaricata* *Tapirira guianensis*
Pteris vittata L.	*Tapirira guianensis*
Pteridium aquilinum (L.) Kuhn var. *arachnoideum* (Kaulf.) Brade	*Copaifera langsdorffii* *Endlicheria paniculata* *Eugenia ligustrina* *Inga affinis* *Tapirira guianensis*
Thelypteris burkartii Abbiatti	*Copaifera langsdorffii*
Thelypteris conspersa (Schrad.) A. R. Sm.	*Aspidosperma cylindrocarpum* *Copaifera langsdorffii* *Endlicheria paniculata* *Eugenia florida* *Inga affinis* *Luehea divaricata* *Tapirira guianensis*
Thelypteris dentata (Forssk.) E. St. John	*Copaifera langsdorffii* dead tree *Endlicheria paniculata* *Eugenia florida* *Eugenia ligustrina* *Tapirira guianensis*
Thelypteris hispidula (Decne) C.F. Reed	*Aspidosperma cylindrocarpum* *Copaifera langsdorffii* dead tree

TABLE 7. Continued.

Fern species	Host tree
	Endlicheria paniculata
	Eugenia ligustrina
	Inga affinis
	Luehea divaricata
	Tapirira guianensis
Thelypteris interrupta (Willd.) Iwats.	*Aspidosperma cylindrocarpum*
	Eugenia florida
	Eugenia ligustrina
	Tapirira guianensis
Thelypteris mosenii (C. Chr.) C.F. Reed	dead tree
Thelypteris opposita (Vahl) Ching	*Aspidosperma cylindrocarpum*
	Copaifera langsdorffii
	Coussarea hydrangeaefolia
	dead tree
	Endlicheria paniculata
	Eugenia florida
	Eugenia ligustrina
	Inga affinis
	Luehea divaricata
	Tapirira guianensis
Thelypteris patens (Sw.) Small	*Aspidosperma cylindrocarpum*
	Copaifera langsdorffii
	Luehea divaricata
	Tapirira guianensis
Thelypteris sp.	*Coussarea hydrangeaefolia*
	Eugenia florida
	Eugenia ligustrina
	Luehea divaricata
	Tapirira guianensis

temperature can participate in the spore dispersion (Page, 1979) and several factors act in the trees, modifying their barks and preparing them to shelter epiphytes (Barkman, 1958). Among them are light affecting the temperature and evaporation, rainfall, atmospheric humidity, and characteristics of the trees such as water and vapour capacity of bark, colour influencing the warmth capacity, hardness, presence of fissures, acidity and chemical composition of the bark. As no association between the size of this spore bank and angiosperm species, tree dimensions or chemical composition of the bark was observed, perhaps the wealth or poverty of these trees in relation to the number of viable spores depends on the dispersal spore processes as an important factor. As a consequence of the casual spore dispersion pattern, the bark spore bank has a random distribution among the trunks. The influence of the physical characteristics of the outer bark needs to be studied for a complete understanding of this bank.

The data obtained in this study show that barks retain viable spores that can participate in the regeneration process and population dynamics of the environment. Probably the spores with greater longevity could participate in

TABLE 8. Sporophyte frequency (mean percentage) registered in barks extracted from angiosperms occurring in the gallery forest of the Ecological Station of Panga. Values were calculated in relation to the total number of sporophytes formed in the replications. Data obtained two (February collection) or three months (March and September collections) after the collection of barks.

	Fern species									
	Thelypteris sp.		*Pteridium aquilinum*		*Pityrogramma calomelanos*		*Pityrogramma trifoliata*		*Pteris vittata*	
Angiosperm Species	Feb98	Sep98	Feb98	Sep98	Feb98	Sep98	Feb98	Sep98	Feb98	Sep98
Copaifera langsdorffii Desf.	43.14	88.93	2.56	5.12	2.56	5.95	0.78	0.00	0.00	0.00
Eugenia ligustrina Miq.	59.79	66.67	0.00	25.00	14.58	8.33	3.33	0.00	0.00	0.00
Endlicheria paniculata (Spreng.) Macbride	73.28	86.60	2.50	1.47	4.50	9.31	0.00	2.60	0.00	0.00
Eugenia ligustrina	84.25	91.67	1.78	0.00	4.70	8.33	0.00	0.00	0.00	0.00
Aspidosperma cylindrocarpum Muell. Arg.	52.50	94.44	0.00	0.00	10.71	5.56	5.00	0.00	0.00	0.00
Tapirira guianensis Aubl.	77.38	80.32	0.00	1.92	2.78	14.98	0.00	0.00	0.00	0.00
Coussarea hydrangeaefolia Benth. & Hook. f.	29.16	75.00	0.00	0.00	25.00	0.00	4.17	0.00	0.00	0.00
Eugenia florida DC.	35.00	93.06	0.00	0.00	8.33	6.94	0.00	0.00	0.00	0.00
Inga affinis DC.	81.25	45.00	0.00	0.00	0.00	17.50	6.25	0.00	0.00	0.00
Luehea divaricata Mart.	100.00	98.75	0.00	0.00	0.00	1.25	0.00	0.00	0.00	0.00
dead tree	0.00	94.44	0.00	0.00	0.00	5.56	0.00	0.00	0.00	0.00
Tapirira guianensis	50.00	88.75	0.00	0.00	8.34	11.25	0.00	0.00	0.00	0.00
Aspidosperma cylindrocarpum	66.67	95.58	0.00	0.00	0.00	4.42	0.00	0.00	0.00	0.00
Tapirira guianensis	0.00	66.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33
Luehea divaricata	25.00	75.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

the recomposition processes mentioned above when the tree falls on the soil. In this sense, the participation of the spores included in the soil in these processes can be higher than that included in the barks. The greater quantity of spores on the soil surface and the infrequent fall of trees in the studied forest make the establishment of gametophytes and sporophytes arising from the soil spore bank faster than those originating from bark spore bank.

The existence of viable spores of terrestrial species on tree bark does not answer an important question—Why do terrestrial species not establish themselves on trees?

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The Occurrence of *Trichomanes godmanii* (Hymenophyllaceae) on *Welfia georgii* (Arecaceae) at the La Selva Biological Station, Costa Rica

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ABSTRACT.—Field observations suggested that the epiphytic fern *Trichomanes godmanii* occurred more frequently and abundantly on the trunks of the palm *Welfia georgii* than on the trunks of dicotyledonous trees. We tested this observation statistically by randomly selecting 25 individuals of *W. georgii* and the nearest dicotyledonous tree of similar dbh, for a total of 50 trunks. For each trunk up to a height of three meters, we recorded the presence or absence of *T. godmanii* and, if present, we visually estimated percent cover using a ranked scale. We found that the fern occurred more frequently and abundantly on the palm than on dicotylendonous trees. No relationship was found between the diameter of the trunks and vegetative cover. This is one of the few host-specific preferences recorded among epiphytic ferns. We cannot fully explain why the fern occurs more frequently and abundantly on the trunks of *Welfia georgii* instead of dicot trees, but the fern's adhesive hairs on its rhizomes and petioles probably help attachment to the smooth trunk of the palm.

The epiphytic habit is prominent in pteridophytes. Of the approximately 9,000 species of pteridophytes considered by Kress (1986, 1990) worldwide, about one-third were epiphytes. In Mesoamerica about 36% of the pteridophyte species are epiphytic (compiled from Moran and Riba, 1995), and in Costa Rica at the Monteverde Cloud Forest Reserve and the La Selva Biological Station, epiphytic species compose 49% and 42% of the pteridoflora, respectively (Grayum and Churchill, 1987; Nadkarni and Wheelwright, 2000). Despite the prominence of epiphytism in pteridophytes, little work has been done on documenting species-specific host associations. Most reports of associations consist of casual observations, such as those reported for fern epiphytes largely or entirely restricted to the root mantles of tree ferns—epiphytes such as *Tmesipteris* in Australasia (Brownsey and Smith-Dodsworth, 1989), and *Blechnum fragile* (Liebm.) C. V. Morton & Lellinger, *Costaricia werckleana* H. Christ, *Terpsichore lehmanniana* (Hieron.) A. R. Sm., *T. semihirsuta* (Klotzsch) A. R. Sm., and *Trichomanes capillaceum* L. in Mesoamerica (Moran and Riba, 1995, p. 399). The only statistical demonstration of species-specific host relationships in ferns was by Moran *et al.* (2003). They studied low-trunk epiphytic ferns on tree fern root mantles versus angiosperms at four sites in Costa Rica. They found that of the 31 species that occurred frequently enough in their samples to be tested statistically, 11 (35%) occurred more frequently on tree fern root mantles.

The present study was prompted by an observation made by Grayum and Churchill (1989) at the La Salva Biological Station in Costa Rica about the frequency of occurrence of *Trichomanes godmanii* Hook. on the trunks of the palm *Welfia georgii* H. A. Wendl. *ex* Burret. They observed that *T. godmanii* was frequently found as a low-trunk epiphyte on the palm—a palm ubiquitous at the La Selva—and that it is one of the few epiphytes seen on the palm (Fig. 1). This observation was subsequently confirmed by the senior author at the La Selva Biological Station and at other nearby lowland forests in Costa Rica. We decided to test these observations by sampling and analyzing the results statistically. We tested the following two null hypotheses: first, that there is no difference in the frequency of occurrence of *T. godmanii* on *Welfia georgii* vs. dicotyledonous trees; second, that there is no difference in the abundance, expressed as percent vegetative cover, of *T. godmanii* on *Welfia georgii* versus dicotyledonous trees.

Besides these two hypotheses about host preference, we examined the possible influence of the palm's dbh on the percent cover of *Trichomanes godmanii.* We expected no influence because the age of the palm is not correlated with the diameter of its trunk (Rich, 1986). Therefore, larger diameter trunks would not necessarily be available for a longer time for the fern to colonize and form a greater percent cover. The null hypothesis we tested was that of no correlation between percent vegetative cover of the fern and the trunk diameter of the palm.

Methods

The La Selva Biological Station is located in Heredia Province, at the confluence of the Puerto Viejo and Sarapiquí rivers, near Puerto Viejo de Sarapiquí, on the Caribbean side of Costa Rica, 10°26′N, 83°59′W. The elevation is about 50 m, and the vegetation is relatively aseasonal, tropical wet forest, with an average annual rainfall of 4,000 mm (McDade and Hartshorn, 1994).

We sampled along an established trail called the *Camino Experimental Sur* beginning at a point where it meets the *Sendero Tres Ríos* in front of the clearing that harbors the laboratory buildings. We sampled 25 pairs of trees, each pair consisting of one *Welfia georgii* and one angiosperm. We first sampled a palm and then selected the nearest dicotyledonous tree of similar (± 10 cm) dbh. We sampled pairs to control for microclimatic effects, and we sampled trunks of similar dbh to control for differences in presence or percent cover that might be associated with trunk width. Only the lower three meters of the trunks were sampled. When the fern was present, percent vegetative cover on each trunk was visually estimated and then scored using a ranked scale where 0 = absent, 1 = 1–10% cover, 2 = 11–25%, 3 = 26–50%, 4 = 51–75%, 5 = 76–100%.

To test the first hypothesis (no difference in the frequency of occurrence of *Trichomanes godmanii* on *Welfia georgii* versus dicot trunks), we used Fisher's Exact Test (Langley, 1971). This test is a 2×2 contingency table that should be used instead of a chi-square contingency table when N is between 8 and 50 (we had $N = 50$; i.e., the 25 pairs). The probabilities were calculated for a

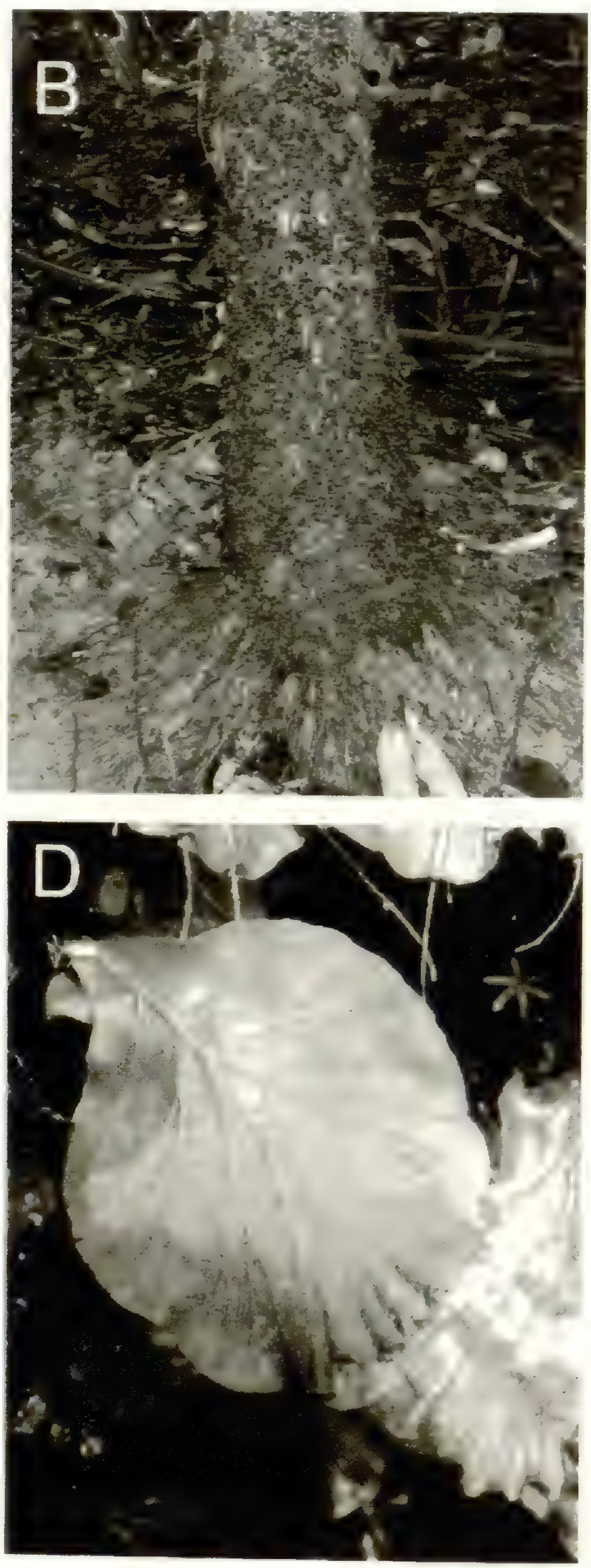

FIG. 1. A. *Welfia georgii* without *Trichomanes godmanii*, showing smooth surface of trunk. B. Trunk of *Welfia georgii* covered with *T. godmanii*. C. Close up of *T. godmanii* colony. D. Fertile frond of *T. godmanii* (about 1 cm long). All photographs taken at the La Selva Biological Station, Costa Rica.

TABLE 1. Contingency table showing the occurrence of *Trichomanes godmanii* on the trunks of *Welfia georgii* and dicotyledonous trees at the La Selva Biological Field Station, Costa Rica. According to a Fisher's exact test, *T. godmanii* occurred more frequently on the trunks of *W. georgii* ($P = 0.0014$).

	Dicot	*W. georgii*	Total
Absent	20	8	28
Present	5	17	22
Total	25	25	50

two-tailed test because we were interested in whether *T. godmanii* occurred more frequently on either *Welfia georgii* or angiosperms trunks. To test the second hypothesis (no difference in the percent vegetative cover of *T. godmanii* on *Welfia georgii* versus dicot trunks), we used a Wilcoxon/ Kruskal-Wallis rank sum test. Included in this test were only those trunks where the fern was present (17 palms, 5 dicots). For the third hypothesis (no correlation between percent vegetative cover of *T. godmanii* with the dbh of the palm trunks), we included only those 17 trunks where the fern was present. We then tested for a correlation between dbh of the palm and percent cover of the fern using Spearman's rank coefficient. The significance level for all three hypotheses was set at $P < 0.05$. The statistical tests described in this paragraph were performed using the JMP statistical package, version 3.3.2 (Sall and Lehman, 1996).

Several species of *Trichomanes* with leaves less than 3 cm long occur at the La Selva Biological Station, and these can be easily confused with *T. godmanii* (Grayum, 1989). During sampling we examined suspected individuals of *T. godmanii* for the presence of cross-connections between the false veins. This characteristic distinguishes *T. godmanii* from all other similar small species of *Trichomanes* (Wessels Boer, 1962). Other characteristics helpful in identifying the species were glabrous lamina margins (i.e., without black paired or stellate hairs) and green-margined involucres (not black margined; Fig. 1D). At the La Selva Biological Station, none of the other small species of *Trichomanes* form large extensive mat-like colonies that *T. godmanii* does on the trunks of *Welfia georgii*.

RESULTS

In the 25 paired samples, *Trichomanes godmanii* was present on 17 palms and 5 dicot trees. The mean dbh of the palms sampled in this study was 17 cm (s.d. 2.2), with a range of 13–25 cm. The mean for the nearest dicot trees of similar dbh was 17.7 cm (s.d. 5.5), with range of 9–26 cm dbh.

Hypothesis 1.—no difference in the frequency of occurrence of *Trichomanes godmanii* on *Welfia georgii* versus dicot trunks. The null hypothesis was rejected ($P = 0.0014$; Table 1). The fern occurs more frequently on the palm.

Hypothesis 2.—no difference in the percent vegetative cover of *T. godmanii* on *Welfia georgii* versus dicot trunks. The null hypothesis was rejected

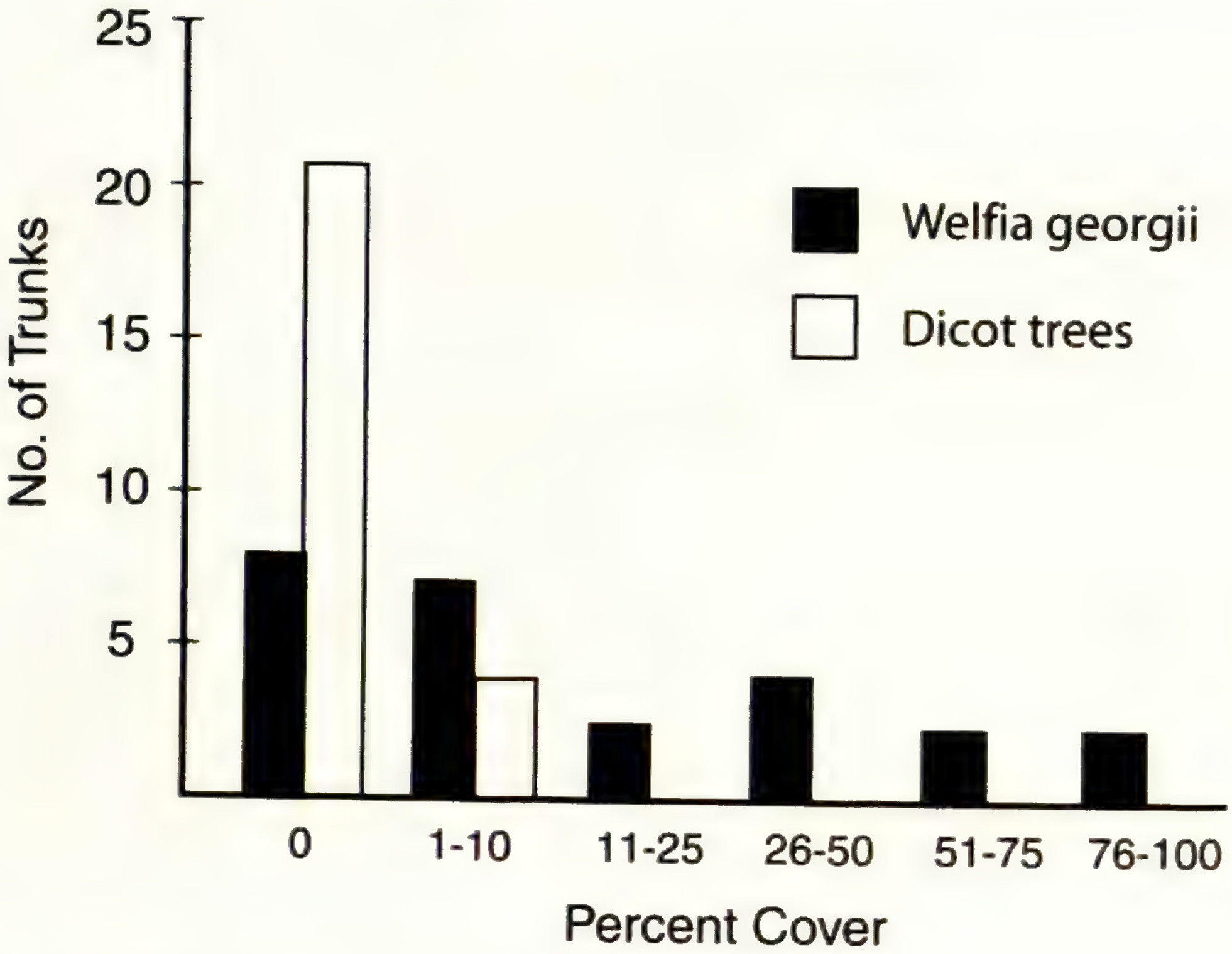

FIG. 2. Comparison of percent vegetative cover of *Trichomanes godmanii* on 25 trunks of *Welfia georgii* and 25 trunks of dicotyledonous trees at the La Selva Biological Station, Costa Rica. According to a Wilcoxon/Kruskal-Wallis rank sum test, *T. godmanii* had greater percent cover on *Welfia georgii* than on dicotyledonous trees ($P = 0.0186$).

($P = 0.0186$; Fig. 2). The fern is more abundant (higher percent cover) on the palm.

Hypothesis 3.—no correlation between percent vegetative cover of *T. godmanii* with the dbh of the palm trunks. The null hypothesis was retained ($r_s = 0.37$; $P = 0.149$). There was no correlation between the abundance of fern and the dbh of the palm.

DISCUSSION

The results support the observations of Grayum and Churchill (1989) that *Trichomanes godmanii* occurs more frequently on the trunks of *Welfia georgii* than on dicot trees (hypothesis 1). This is one of the few host-specific relationships that have been documented statistically in ferns (for others see Moran *et al.* 2003).

Trichomanes godmanii seems to be one of the few ferns capable of colonizing the trunks of *Welfia georgii*. Grayum and Churchill (1989) noted that an unnamed species of *Elaphoglossum* also occurred rarely on the trunks, but we did not find this species in or outside our samples. The only other fern we found on the palm was *Trichomanes* (sect. *Didymoglossum*) *angustifrons* (Fée) W. Boer. It occurred as isolated individuals near the base of three trunks.

The results also showed that *Trichomanes godmanii* was not only more frequent on the palm, but also more abundant (hypothesis 2). The colonies could be so dense that the surface of the trunk was obscured. When present, they always occurred around the base of the trunk (Fig. 1B) and diminished upward, but in some cases dense colonies extended five meters above the ground (at La Selva the palm can attain a height up to 23 meters; Rich, 1986). The large extensive colonies of the fern (Fig. 1B) are formed by the plants' long-creeping rhizomes that occasionally branch and run horizontally or upward around the trunk.

There was no correlation, however, between percent cover of the fern and dbh of the palm (hypothesis 3). Like many palms, *Welfia georgii* begins vertical growth with a stem girth that is sufficient to support its maximum height, and it maintains the same or nearly same width as it grows taller (Rich, 1986). Thus wider trunks do not necessarily represent older individuals that have been available for colonization for a longer time by epiphytes. Given this, one would expect an equal amount of the fern in terms of percent cover on both narrow and wide diameter trunks, and this is what we found.

Why is *Trichomanes godmanii* most frequent and abundant on *Welfia georgii*? Perhaps allelopathy plays a role, with the palm trunk presenting a chemical that inhibits epiphytes other than the fern. This idea, however, cannot be assessed because allelopathy has never been investigated in the trunks of *W. georgii* nor any other palm (Andrew Henderson, pers. com.). More likely in promoting growth of the fern is the smooth texture of the palm trunk. This might favor the fern two ways. First, it could hinder other epiphytes from establishing and attaching the trunk. This might be because of the smooth surface itself or because such surfaces retain less water or nutrients than rougher surfaces. In either case, fewer epiphytes would free the fern from competition, allowing it more space, light, and nutrients. Second, unlike other epiphytes, the smooth surface of the palm might be an easy substrate for *T. godmanii* to grasp. Like all members of *Trichomanes* sect. *Didymoglossum*, *T. godmanii* is rootless, but its rhizomes, petioles, and sometimes basal portions of the midrib, bear abundant specialized hairs called "adhesive hairs" (Schneider, 2000). These usually form a dense mat surrounding the rhizome and are dark, stiff, and several-celled. They have a cuticle (as do the rhizomes) and apparently do not absorb water or mineral nutrients, but they might hold water by capillary action and gradually release this water to the lamina as it dries. Many species of *Trichomanes* absorb water and mineral nutrients directly through their leaves, which are only one cell layer thick between the veins and lack a cuticle or nearly so (Haertel, 1940). The main function of the hairs, however, appears to be for attachment. The presence of these numerous hairs greatly increases the surface area for clinging to the substrate. In some species of *Trichomanes* sect. *Didymoglossum*, the adhesive hairs branch or enlarge at the tip when they touch the substrate (Duckett *et al.*, 1996), increasing adhesive ability. *Trichomanes godmanii* has such branched or swollen hairs (pers. obs.) that would facilitate its colonizing smooth surfaces. *Trichomanes angustifrons*, the only other fern we found on the trunks, is also

a member of sect. *Didymoglossum* and has the adhesive hairs, suggesting the importance of this type of indument for growing on smooth surfaces. Nevertheless, adhesive hairs cannot be the only reason why *T. godmanii* flourishes on palm. Four other species of *Trichomanes* sect. *Didymoglossum* occur at the La Selva Biological Station (Grayum and Churchill, 1989), and although they have adhesive hairs, only one of them (*T. angustifrons*) was found on the palm. All of the other four grow primarily on dicots. Thus, although adhesive hairs probably play an important role, they are not the entire reason why *T. godmanii* prefers the trunks of *Welfia georgii*.

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A New Species of *Adiantum* (Pteridaceae) from Thailand

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ABSTRACT.—A new species, *Adiantum thongthamii* (Pteridaceae), known only from a small island in southeastern Thailand is described and illustrated.

Adiantum is a genus of ca 150 species widely distributed pantropically but extending as far as southern South America, New Zealand, Newfoundland, Alaska, and northeastern Asia, (Tryon & Tryon, 1982; Mabberley, 1997). Tagawa & Iwatsuki (1989) record ten species of Adiantum from Thailand.

During a field trip to southeastern Thailand in March 2002, an eleventh species, *Adiantum thongthamii*, was found in Koh Chang, a small island close to Cambodia.

Adiantum thongthamii Suksathan *sp. nov.* TYPE:—Thailand: Trat Province: Koh Chang, 600 m alt., 24 March 2002, *P. Suksathan 3303* (holotype: QBG; isotypes: AAU, L, US). **Figs. 1, 2.**

Species nova, Adianto erylliae C.Chr. & Tard. affinis a qua differt squamibus rhizomatis concoloribus rufis, foliis dense pubescentibus, rachidi non prolifera, petiolulis ca 1 mm, pinnis late flabellatis usque rotundatis, coriaceis, soris minoribus ca 1 mm latis, 8–21 in quaque pinna, pseudoindusiis minoribus obovatis 0.5–1.0 mm latis.

Plants terrestrial. Rhizomes short-creeping, erect to sub-erect, ca 5 mm diam; scales copious, narrowly lanceolate to linear, 4–5 × 0.1–0.9 mm, lustrous, concolorous, reddish brown, margins entire to sparsely minutely toothed in the upper half. Fronds monomorphic, simply pinnate, with or without a (smaller) conform terminal pinna, 7–20 cm long; stipes 3–10 cm long, lustrous, reddish brown to nearly black, sparsely to densely covered with grayish to reddish brown multicellular hairs; the hairs, spreading, 1.5–2 mm long, decreasing to ca 1 mm long toward the apex. Lamina oblong, to 2.5 cm wide; rachis with indument similar to the upper part of the stipe, neither prolonged nor proliferous; pinna pairs 3–6 (–9), opposite to alternate, basal pinnae often slightly reduced in size, upper pinnae rarely gradually reduced in size. Pinnae; stalks ca 1 mm long, with short hairs, flabellate-cuneate to flabellate-truncate or suborbicular, 7–15 × 9–20 mm, green when young becoming dark bluish green with age; distally lobed to 1/3 the length of the pinna, the sinuses typically narrow, lobes rounded to truncated, entire in sterile pinnae, coriaceous, idioblast absent, both surfaces densely hairy; the hairs ca 1 mm long, adaxial hairs gray, abaxial hairs

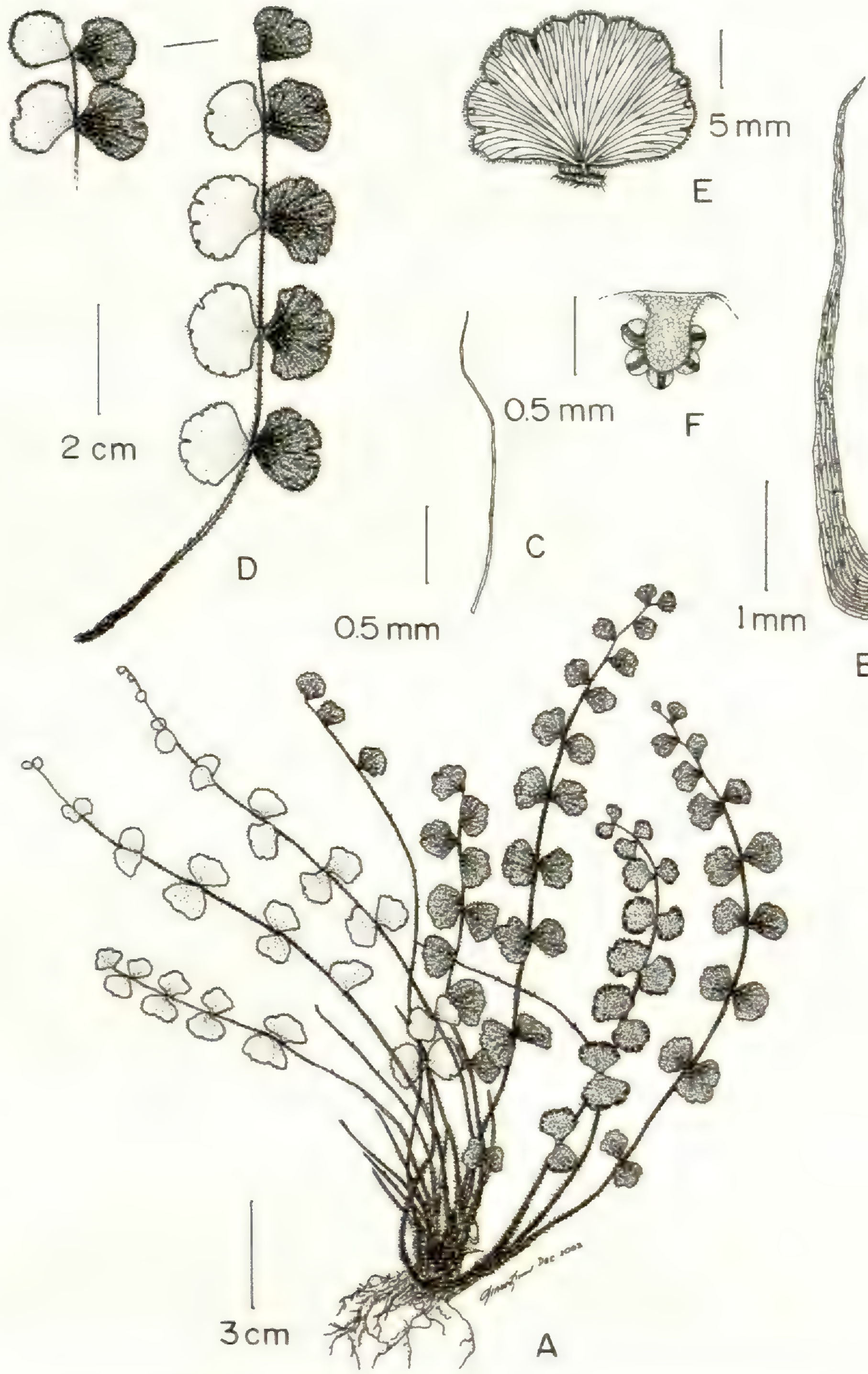

FIG. 1. *Adiantum thongthamii* Suksathan. A) Habit; B) rhizome scale; C) stipe hairs; D) frond and frond apex; E) pinna, abaxial surface with hairs removed; F) sorus with hairs removed. Drawn from the holotype (*P. Suksathan 3303*, QBG).

FIG. 2. Habit view of *Adiantum thongthamii* Suksathan at the type locality.

brown; veins free, forked, prominulous on lower surfaces, in dry specimens. Sori round, 8–21 per pinna; pseudoindusia small, obovate, 0.5–1 mm long, margin entire, hairy. Spores trilete, dark yellow to brown, the surfaces rugose, ca 50 mm.

DISTRIBUTION.—Known only from the type locality at Koh Chang (12° 0′ N, 102° 22′ E), an island in SE Thailand.

HABITAT.—Occurs in full sun-xeric habitat on exposed sandstone outcrops along the Khao Laem mountain ridge between 500–640 m. *Adiantum thongthamii* was found growing with *Melastoma* spp. (Melastomataceae), *Nepenthes* spp. (Nepenthaceae), *Doritis* spp. (Orchidaceae), *Adiantum capillus-veneris* L. (Pteridaceae), *Selaginella siamensis* Hieron. (Selaginellaceae), and others.

Adiantum thongthamii is very distinct from other known species of *Adiantum* and is easily recognized by its once pinnate fronds with 3–6 (–9) pairs of broadly fan-shaped pinnae and by its dense wooly pubescence. *Adiantum thongthamii* differs from *A. erylliae* C. Chr. & Tard., and *A. capillus-junonis* Ruprecht by having hairy fronds and many smaller sori per pinna (8–21 versus 2–7) and from *A. caudatum* L. by its concolorous scales and pinna shape (broadly fan-shape versus parallelogram-shaped in *A. caudatum*).

Tryon and Tryon (1982) divided the genus *Adiantum* into eight groups based on morphology. *Adiantum thongthamii* appears to be belong to the *A. philippense* Group, in having simply pinnate fronds, flabellate segments, and free veins. The subdivision by Tryon and Tryon has, however, strongly emphasizes the American species and does not include all taxa in the genus. Some species are also placed with uncertainty. Nevertheless, ongoing work at the DNA level should hopefully reveal more insight into natural groups and species relationships in the genus (A.R. Smith, pers. com.).

The species is named in honor of Associate Prof. ML Charuphant Thongtham of Thailand, an expert pterido-horticulturist who inspired my interest in ferns and plants in general.

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Asplenium ceterach and *A. octoploideum* on the Canary Islands (Aspleniaceae, Pteridophyta)

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ABSTRACT.—Isozyme and plastid DNA analysis prove that true *A. ceterach* occurs on the Canary Islands, in addition to *A. aureum* and an octoploid taxon. Combining morphological and cytological observations leads to correct determination, but the exospore length alone also allows reliable identification of these Canarian species. Our allozyme data suggest that the Canarian *A. ceterach* population is not completely genetically isolated from the European ones. The holotype of *Ceterach aureum* var. *parvifolium*, formerly regarded as an octoploid taxon, proved to be *A. ceterach*, leaving the octoploid without a correct name. The recently described *A. octoploideum* shows monomorphic, presumably fixed heterozygosity for a combination of the patterns seen in *A. ceterach* and *A. aureum* at four loci (*Aat*, *Skdh*, *Me*, and *Pgi-2*) confirming its allo-octoploid nature. It most probably originated by chromosome doubling in a tetraploid hybrid between *A. aureum* and *A. ceterach* or via the union of their unreduced gametes. *Pgi-2* indicates multiple origins of the allo-octoploid, implicating recurrent gene flow from tetraploids to octoploids.

Asplenium subgenus *Ceterach* (Willd.) Bir *et al.* is a small group of about nine fern taxa within the large (720 species), subcosmopolitan genus *Asplenium* L. (Kramer and Viane, 1990). This subgenus contains xerophytic rock ferns with the dorsal side of the lamina densely covered with reddish-brown scales (= paleae). Van den heede *et al.* (2003) have shown that the group must be restricted to the Eurasian and Macaronesian species.

Ever since the description of *Asplenium aureum* Cav. from Tenerife by Cavanilles (1801), it has been unclear how many "*Ceterach*" species are extant in the Canarian Archipelago, and whether the "European" *A. ceterach* L. (Syn.: *Ceterach officinarum* Willd.) occurs in Macaronesia (Table 1). This confusion was caused by the lack of distinctive characters to distinguish both species. Cavanilles (1801) and Bory de St. Vincent (1802) mentioned only the much larger size of *A. aureum* compared to that of *A. ceterach*. Willdenow (1810) introduced the concept of "toothed scales" as a diagnostic feature, whereas Milde (1865) claimed that "Cuticularstreifen" (cuticular lines or ridges on the periclinal cell walls of the scales) could be used to distinguish *A. aureum* from

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TABLE 1. Taxa covered by names found in the literature. Abbreviations used: *A. cet.* = *A. ceterach*, *A. aur.* = *A. aureum*, *A. lol.* = *A. lolegnamense*, *A. par.* = "*A. parvifolium sensu Vida and Reichst.*" = *A. octoploideum*. ■: the filled symbol indicates that the taxon was included in this author's concept of the species mentioned in column 2; □: symbol indicates that this taxon was included in the author's concept of the species mentioned in column 2 prior to its formal description.

		Taxa included in this name			
Literature reference	Name used or published	*A. cet.*	*A. aur.*	*A. lol.*	"*A. par.*"
Linnaeus 1753	*A. ceterach*	■			
Cavanilles 1801	*A. aureum*		■		
Bory de St. Vincent 1802	*A. ceterach*	■			
	A. latifolium		■		
Desvaux 1827	*C. aurea*		■		
von Buch 1828 ["1825"]*	*C. aureum*		■		
Moore 1857	*C. aureum*		■		
	C. officinarum	■			
Lowe, E. J. 1858	*C. officinarum*	■		□	
Hooker 1860, 1861	*A. ceterach*	■			
	A. ceterach var. *aureum*		■	□	
Bolle 1864	*C. aureum*		■		
	C. officinarum	■		□	□
Milde 1866b, 1867a,b	*C. aureum*		■	□	
	C. officinarum	■		□	□
Kuhn 1868	*C. aureum*		■	□	
Sauer 1880	*C. aureum*			□	
	C. officinarum	■			
Luerssen 1889	*C. officinarum*	■	■	□	□
Schneider 1892	*A. ceterach*	■			
	A. ceterach var. *aureum*		■	□	
Christ 1897	*C. aureum*		■		
	C. officinarum	■		□	
Burchard 1929	*C. aureum*		■		
	C. officinarum	■			□
Chevalier 1935	*C. officinarum*	■			
	C. officinarum var. *aureum*		■	□	
Tardieu-Blot 1946	*C. officinarum*	■	■	□	
Copeland 1947	*C. aureum*		■		□
	C. officinarum	■			
Manton 1950	*C. aureum*		■		
	C. officinarum	■			
Romariz 1953	*C. aureum*		■	□	
Lems 1958, 1960	*C. aureum*		■	□	
	C. officinarum	■			□
Dansereau 1961	*C. aureum*	■	■	□	
Fabbri 1965	*C. aureum*		■		
	C. officianarum	■			
Kunkel 1965	*C. aureum*		■	□	□
Benl and Kunkel 1967	*C. aureum* var. *aureum*		■		
	C. aureum var. *parvifolium*	■			■
Lid 1967	*C. aureum*	■	■		
Hansen 1969	*C. aureum*			□	
Benl and Sventenius 1970	*C. aureum* var. *aureum*		■		
	C. aureum var. *parvifolium*	■			■

TABLE 1. Continued.

Literature reference	Name used or published	Taxa included in this name			
		A. cet.	*A. aur.*	*A. lol.*	*"A. par."*
Kunkel 1971	*C. aureum* var. *aureum*		■		
	C. aureum var. *parvifolium*	■			■
Hansen and Sunding 1979	*C. aureum* var. *aureum*		■	□	
	C. aureum var. *parvifolium*	■			■
Reichstein 1984	*C. aureum* var. *aureum*		■		
	C. aureum var. *parvifolium*				■
	C. offinarum	■			
Bir et al. 1985	*A. aureum*		■	□	■
	A. ceterach ssp. *ceterach*	■			
Manton et al. 1986	*C. aureum* var. *aureum*		■		
	C. aureum var. *parvifolium*				■
Gibby and Lovis 1989	*C. lolegnamense*			■	
Ormonde 1990	*C. aureum* var. *aureum*		■		
	C. aureum var. *madeirense*			■	
	C. aureum var. *parvifolium*	■			■
Viane and Reichstein 1992	*A. parvifolium*				■
	A. lolegnamense			■	
Griffiths 1997	*A. aureum*		■	■	
	A. ceterach	■			
Hoshizaki and Moran 2001	*C. aureum*		■	■	■
	C. officinarum	■			

* According to Stafleu and Cowan (1976) this book was only published after 28 May 1828, it is not clear whether Link or von Buch made the combination "*C. aureum*", which is, in any case, antedated by Desvaux (1827).

its continental counterpart, though he soon (Milde 1866a, 1866b, 1867a, 1867b) cast doubt on the utility of this character. As early as May 1866, Milde admitted that "die Cuticularstreifen, welche die Spreuschuppen von *C. aureum* stets zeigen, fand ich nun auch an exemplaren, die sich von *C. officinarum* nicht unterscheiden liessen." Finally, he came to the conclusion that the character was useless to discriminate *A. ceterach* from *A. aureum* (Milde, 1867b). Nevertheless, Bornmüller (Plantae exsicc. Canarienses–1901), and Benl and Kunkel (1967) heavily relied on this character to recognize taxa. Since 1970, chromosome numbers were used to distinguish species in this group (T. Reichstein, pers. comm.), and morphological characters became less important (e.g., Bir *et al.*, 1985; Manton *et al.*, 1986; Gibby and Lovis, 1989).

In 1967, Benl and Kunkel considered all Canarian plants that looked like *A. ceterach* to be a dwarfed variety of *A. aureum*. Unfortunately, their variety *Ceterach aureum* (Cav.) Desv. var. *parvifolium* Benl and G.Kunkel was published without cytological information. In March 1967, T. Reichstein collected living "*A. ceterach*" on Gran Canaria, and sent material for chromosome counts to G. Vida. In 1970, these plants were found to be octoploid, but because good cytological photographs were lacking the results were not published (T. Reichstein, pers. comm.). From then onwards, but without studying the type of *A. parvifolium* (Benl and G.Kunkel) Vida and

Reichst., octoploid status was attributed to it. To clarify the origin of *A. parvifolium*, a hybridization program was started by G. Vida in Budapest; results were partly published in Manton *et al.* (1986). Meanwhile, T. Reichstein had informed many pteridologists about the putative allo-octoploid nature of *A. parvifolium* and briefly mentioned it in Hegi (1984).

To date two cytologically different endemic species are generally accepted to occur on the Canary Islands: *A. aureum* and *A. parvifolium*. *Asplenium aureum* was found to be tetraploid by Manton (1950). Vida and Reichstein (Vida, 1972; Viane and Reichstein, 1992) suggested *A. aureum* to be allotetraploid, which was confirmed by ITS analysis (Van den heede *et al.*, 2003). The name *A. parvifolium* was used for the allo-octoploid that probably formed by chromosome doubling of the tetraploid hybrid between *A. aureum* and *A. ceterach* (Vida, 1972; Viane and Reichstein, 1992). After 1970, all small Canarian plants that looked like *A. ceterach* were considered to be a) *A. parvifolium* and b) octoploid. According to Manton *et al.* (1986) "*C. officinarum* is not positively recorded from Macaronesia, but its former presence, at least in the Canaries, is suggested by the morphology of some representatives of *C. aureum sens. lat.* from these islands."

Within *A. ceterach sensu lato* three cytotypes are known, and according to the Biological Species Concept (Mayr, 1942, 2000; see review in King, 1993), autopolyploids should be considered separate species because they produce sterile hybrids with their parents from which they are reproductively isolated. Diploid *A. javorkeanum* Vida [Syn.: *A. ceterach* ssp. *bivalens* (D.E. Mey.) Greuter and Burdet; *C. officinarum* Willd. ssp. *bivalens* D.E. Mey.] is known from Albania, Bulgaria, Croatia, Greece, Hungary, Italy, Romania, and Slovenia (Vida, 1963; Reichstein, 1984), and should be looked for in northern Algeria and Turkey, because the triploid hybrid *A.* ×*mantoniae* Váróczy and Vida was found there (Greuter, 1980; Viane *et al.*, 1996). Tetraploid (Manton, 1950; Vida, 1963) *A. ceterach* [Syn.: *C. officinarum* Willd. ssp. *officinarum*] is supposed to have originated via chromosome doubling in *A. javorkeanum*; its autopolyploid status was confirmed cytologically by Rasbach *et al.* (1987). The autotetraploid is the more common species, occurring throughout Europe (see maps in Jalas and Suominen, 1972; Pichi Sermolli, 1979; Reichstein, 1984), southwestern Asia and the western Himalayas. *Asplenium ceterach* is more rare in northern Africa (Jahandiez and Maire, 1931; Maire, 1952; Quezel and Santa, 1962; Siddiqi, 1989), but extends into Eritrea and Somalia (Viane *et al.*, 1996), the Arabian Peninsula (Collenette, 1985), and Yemen (Christ, 1900; Wood, 1997). The autohexaploid *A. cyprium* Viane and Van den heede (Syn.: *A. ceterach* ssp. *cyprium* Viane) was described from Cyprus (Van den heede and Viane, 2002; Viane and Van den heede, 2002), and is also known from Greece and Sicily (Viane *et al.*, 1996; Van den heede *et al.*, 2002).

For the biosystematic revision of the *Ceterach* group (Van den heede, 2003), field trips were organized to study the Macaronesian representatives. Plants that we could not distinguish from the European *A. ceterach* were tentatively called *A. parvifolium*, and assumed to be octoploid. To our great surprise many of them turned out to be tetraploid.

In order to clear up the *A. ceterach–A. parvifolium* muddle on the Canarian Archipelago, we studied type material, and cytologically checked 145 samples from Gran Canaria, La Palma, and Tenerife. Because the type of *A. parvifolium* turned out to be *A. ceterach*, the octoploid taxon needed a new name and was described as *A. octoploideum* Viane and Van den heede (Van den heede and Viane, 2002).

Because electrophoretic analysis of isozymes has been successfully used in studies of reticulate complexes of Pteridophyta (Werth *et al.*, 1985a, 1985b; Werth, 1991; Haufler *et al.*, 1995) and has been applied at population and species levels (see Haufler, 1985b, 1997), we tried this method together with DNA sequencing, to determine whether true *A. ceterach* grows on the Canary Islands. A combination of morphological, cytological, and biogeographical data and isozyme markers can determine whether taxa are auto- or allopolyploid (Crawford, 1985; Haufler, 1985b; Bryan and Soltis, 1987; Weeden and Wendel, 1989; Crawford, 1990; Pryer and Haufler, 1993). An overview of the literature about DNA sequencing in Pteridophyta is given in Van den heede *et al.* (2003).

Material and Methods

Between April 1995 and May 1999, field trips were organized to three Canary Islands from which *A. parvifolium* was known in the literature: Gran Canaria, La Palma and Tenerife. From 145 specimens, fronds with ripe spores were collected by C.V. and R.V, and ecological notes were made. Voucher information (Appendix 1, 2, and 3) is given only for specimens from which we were able to raise progeny and obtain cytological data.

The following localities are shown in Fig. 1:

1) Gran Canaria, S of Moya, "Los Tilos" Reserve, W exposed slopes of Barranco del Laurel, degraded laurel forest, in fissures of volcanic rocks; 28°05′03″N, 15°35′28″W, 600 m alt.
2) Gran Canaria, 4 km from junction Tejeda–San Mateo–Las Mesas, E exposed slopes of "El Nieblo" Nature Reserve, in fissures of volcanic rocks; 28°01′03″N, 15°36′07″W, 1550 m alt.
3) Gran Canaria, lava field near Cueva Corcho, along road GC110 from Artenara to Valleseco, 4 km NW of junction Artenara–Valleseco–Tejeda, in fissures of volcanic rocks; 1350 m alt.
4) Gran Canaria, 900 m S of Valsendero, W exposed cliff sides of narrow gully with laurel forest remnants; 28°02′48″N, 15°34′27″W, 900 m alt.
5) La Palma, c. 3 km E of Tijarafe, Pinar Lomo del Horno; 28°42′N, 17°55′W, 1140 m alt.
6) La Palma, S of Gallegos, Barranco Lomo de los Machines, Laurel forest W of tunnel El Envetadero, E exposed slope; 28°48′N, 17°50′W, 390 m alt.
7) La Palma, volcanic rocks above roadside to Fuencaliente, S of Monte de Luna; 28°31′N, 17°49′W, 710 m alt.
8) La Palma, footpath to Monte de Luna in Pinar S of Flores, in fissures of volcanic rocks; 28°31′N, 17°49′W, 810 m alt.

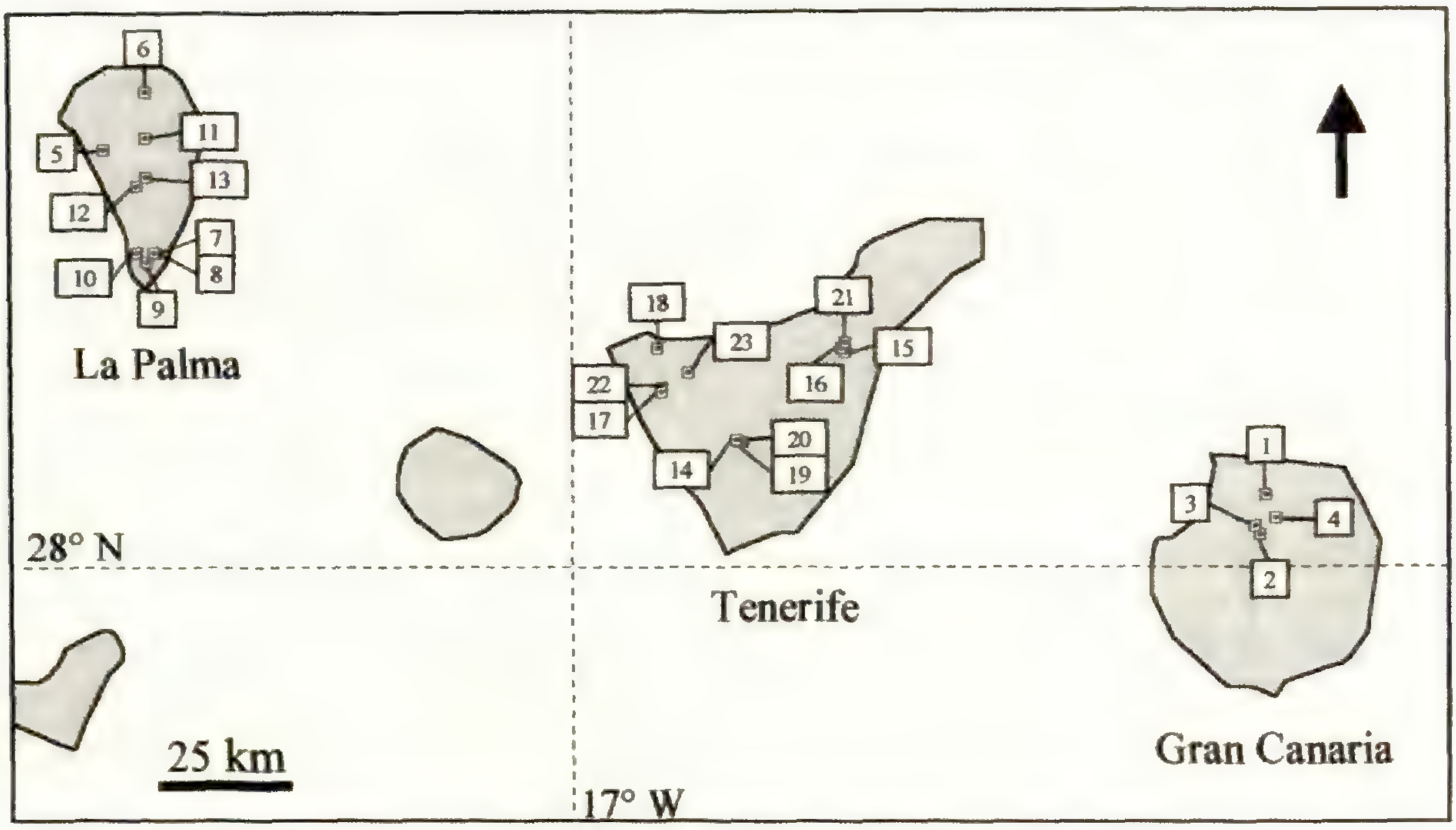


FIG. 1. Map of the western Canary Islands with localities of voucher specimens (see also Appendix 1, 2, and 3).

9) La Palma, along track to Pinar de la Virgen at junction with track to Caldera Los Arreboles, in fissures of volcanic rocks; 28°30′N, 17°50′W, 920 m alt.
10) La Palma, along track to Refugio de Tigalate from Zona Recreativa Fuente de los Roques, above "Malpais" W of Monte de Luna, in fissures of volcanic rocks; 28°31′N, 17°49′W, 1070 m alt.
11) La Palma, Caldera de Taburiente, track from La Cumbrecita to Hoyo de los Pinos, Pinar in Barranco de la Faya, in fissures of volcanic rocks; 28°43′N, 17°50′W, 1200 m alt.
12) La Palma, lava field 2.5 km E of El Paso church, in fissures of volcanic rocks; 28°38′N, 17°51′W, 800 m alt.
13) La Palma, lava field E of El Paso, NE of Montaña Las Moraditas, in fissures of volcanic rocks; 28°39′N, 17°50′W, 800 m alt.
14) Tenerife, along road from Vilaflor to Pico del Teide, ca. 8.1 km from junction Vilaflor–Santiago del Teide–La Orotava, under disc-like, SW exposed volcanic rocks; 28°10′55″N, 16°39′17″W, 1850 m alt.
15) Tenerife, Barranco de las Gambuesas above Arafo, N exposed slopes; 28°20′29″N, 16°26′02″W, 710 m alt.
16) Tenerife, Barranco del Espigon de Tea, NE exposed slopes; 28°20′44″N, 16°26′33″W, 825 m alt.
17) Tenerife, Montaña de la Hoya, ridge S of Las Manchas, above Ermita de la Santa Angel del Guardo, in fissures of volcanic rocks; 28°16′34″N, 16°48′05″W, 1120 m alt.
18) Tenerife, Teno, Barranco head between Tierra del Trigo and Ruigomez, along track above Tierra del Trigo, 1.8 km NW of Ruigomez, NW exposed basaltic slopes; 28°20′48″N, 16°48′28″W, 800 m alt.

19) Tenerife, Pinar above Vilaflor, Bandes de Chasna, c. 2.5 km NNW of Vilaflor, E exposed, in fissures of volcanic (phonolite) rocks; 28°10′48″N, 16°38′36″W, 1880 m alt.
20) Tenerife, Pinar above Vilaflor, W exposed slopes of small Barranco, in fissures of volcanic rocks; 28°10′48″N, 16°38′39″W, 1900 m alt.
21) Tenerife, Barranco de la Piedra Cumplida above (NW) Arafo; 28°21′17″N, 16°26′05″W, 900 m alt.
22) Tenerife, volcanic outcrop along footpath between Santiago del Teide and Arguayo, SE of El Retamar, between Montaña de la Hoya and La Hoya; 28°16″N, 16°48″W, 920 m alt.
23) Tenerife, lava field NW of Montaña de las Flores, "Vuelta Grande", along track from El Portillo del Rastrojo to Llanos del Hospital; 28°18″N, 16°45″W, 1410 m alt.
24) Tenerife, Chio Street, direction Cañadas, Restaurant "De Evora".

Vouchers listed in Appendix 1, 2 and 3, are deposited in the personal herbarium of Viane and Van den heede (including the T. Reichstein herbarium), with duplicates in GENT.

Between 1992 and 2001, R.V., C.V., and W. Bennert gathered additional material in Europe, Madeira, and Turkey (Appendix 4). Voucher information about 108 Cypriot samples is published in Van den heede *et al.* (2002). Our living European and Macaronesian *Asplenium* subg. *Ceterach* collection contained up to 550 specimens.

All material for this study has been cultivated in Ghent University Botanical Garden (Belgium). Spores were sown on agar-solidified medium containing a nutrient solution recommended by Dyer (1979). The cultures were stored in continuous light at room temperature. After formation of mature gametophytes, distilled water was added to achieve fertilization. If necessary, prothallia were transplanted onto fresh agar. Young sporophytes were planted individually in pots kept in a temperate greenhouse (minimum temperature 12°C). An air- and water-permeable soil mixture was required for these xerophytic rock ferns. Full-grown maturity was reached after approximately two years.

For chromosome counts, immature spore mother cells were fixed in the field, or in the greenhouse, using freshly prepared 3:1 absolute ethanol:glacial acetic acid, and stored at freezing-temperature until required. Acetocarmine squash preparations were made as described by Heitz (1925, 1950) and Manton (1950). Photographs were taken with an Olympus BH2 phase contrast microscope. Preparations were made permanent by dehydrating cover slip and slide in graded mixtures of acetic acid and absolute ethanol, followed by mounting in Euparal (T. Walker and H. Rasbach, pers. comm.). All permanent preparations are kept in the Pteridological Section of the Department of Biology at Ghent University. Sixteen cytologically checked plants (five tetraploids identified as *A. aureum*, seven tetraploids identified as "*A. parvifolium sensu* Benl," and four octoploids, (Appendix 1, 2, 3) were used as standards to compare the nuclear DNA content of the remaining specimens by a flow cytometer (Partec PA-1), using the manufacturer's protocol (Partec GmbH, Münster,

Nordrein-Westfalen, Germany). Both nuclei extraction solution and DAPI staining dilution were provided by Partec (Germany).

Methods for making permanent epidermis preparations and for measuring stomatal guard cells and spores, are described by Viane (1990, 1992). For exospore measurements untreated, fresh spores mounted in DePeX were used. Spore size is unaffected by DePeX, whereas in some other mounting media, e.g., glycerin-gelatin (Ormonde, 1990), spores expand by 5–15 %. The values of microcharacters are extracted from our regularly updated database, presently containing 110 different specimens of the European "*A. ceterach*" group, and 56 specimens of the Macaronesian "*A. aureum*" group (raw data available upon request).

Only vigorously growing plants were included in the allozyme study. Fresh leaves from 105 Canarian (Appendix 1, 2, 3) and 220 European and Turkish specimens (Appendix 4) were collected in the greenhouse, where the ferns were growing under the same conditions. Sporulating fronds of similar age were wrapped in wet tissue, stored in plastic bags, and kept refrigerated at 4°C for maximum 0.5–2 days (until extraction). Polyacrylamide gel electrophoresis (PAGE) was performed by C.V. at the laboratory of "General Botany and Nature Management" of the Free University Brussels (Belgium). Starch gel electrophoresis (SGE) was done by S.P. and E.P. in the "Departamento de Biologia Vegetal I" of the Universidad Complutense in Madrid (Spain). All specimens from the Canarian Archipelago were analysed by starch gel electrophoresis.

Equal amounts of tissue and extraction buffer were used to obtain uniform concentrations of extracts. Cooling (4°C) was applied during both homogenization and electrophoresis. Acquaah (1992) was consulted for the Enzyme Commission (E.C.) numbers.

PAGE procedures mentioned in Triest (1989) and Van den heede *et al.* (2002) were used, whereas SGE protocols followed Soltis *et al.* (1983) and Haufler (1985a). In a preliminary survey 19 enzyme systems (G-3PDH, G-6PD, GDH, IDH, MDH, ME, 6-PGD, SkDH, SOD, XDH, ACO, AAT, HK, PGM, β-EST, LAP, ALD, PGI, and TPI) were checked for polymorphism. Because the primary goal of this isozyme research was to test the hypothesis that tetraploid *A. ceterach* occurs on the Canary Islands in addition to *A. aureum* and related taxa, it was necessary to identify unique "marker" alleles characterizing each species or its progenitors. Finally, only five enzyme systems were suitable: aspartate aminotransferase (AAT = GOT, E.C. 2.6.1.1), shikimate dehydrogenase (SkDH, E.C. 1.1.1.25), malic enzyme (ME, E.C. 1.1.1.40), phosphoglucose isomerase (PGI, E.C. 5.1.3.9), and triosephosphate isomerase (TPI, E.C. 5.3.1.1). All pictures and dried gels are kept in the Pteridological Section of the Department of Biology at Ghent University.

Band homologies were determined by running samples side-by-side on the same gel (see Haufler *et al.*, 1995). Allelic variants within loci were distinguished from the products of different loci by assuming that *Asplenium* enzymes conformed to established models of organellar compartmentalization (Gottlieb, 1982; Gastony and Darrow, 1983; Soltis, 1986; Weeden and Wendel, 1989). Presumed loci were numbered sequentially, with the most anodally

(i.e., the fastest band) migrating one designated "1." Similarly, different alleles of the same gene locus (i.e., allozymes, Crawford, 1990) were denoted alphabetically with the most anodal being "*a*."

Sequencing work was done by C.V. at the Jodrell Laboratory in Kew (United Kingdom). Nineteen cytologically and isozymically interesting, vigorously growing plants, including three *A. aureum* specimens (*CV164, CV670, CV712*) from Gran Canaria, Tenerife, and La Palma, one putative *A. ceterach* from Tenerife (*CV187*), and one octoploid from La Palma (*CV709*), were selected to generate DNA sequences from the plastid *trnL-trnF* intergenic spacer. European material for comparison included two *A. javorkeanum* specimens from Italy and Slovenia (*CV14* and *CV85b*), two *A. ceterach* samples from Italy and Cyprus (*CV494* and *CV225*), and a hexaploid *A. cyprium* plant (*CV249*) from Cyprus (see Appendix 4). Sequences of the closely related *A. lolegnamense* (Gibby and Lovis) Viane from Madeira (*CV985 and CV993*), of the less related *A. dalhousiae* Hook. from Ethiopia and Pakistan (*CV318 and TR7634*), and of the more distantly related *A. nidus* L. (*AF425118*), and *A. scolopendrium* L. and *A. unilaterale* Lam. (R. Cranfill, University of California, Berkeley, California, USA, unpublished data) were included as outgroups. The *trnL-F* sequence of a species of *Dennstaedtia* (R. Cranfill, unpublished data) was used to represent a group basal to the Aspleniaceae (e.g., Bower, 1928; Christensen, 1938; Copeland, 1947; Pichi Sermolli, 1977; Kramer and Green, 1990; Hasebe *et al.*, 1995; Pryer *et al.*, 1995). Methods are explained in Van den heede *et al.* (2003).

Results and Preliminary Discussion

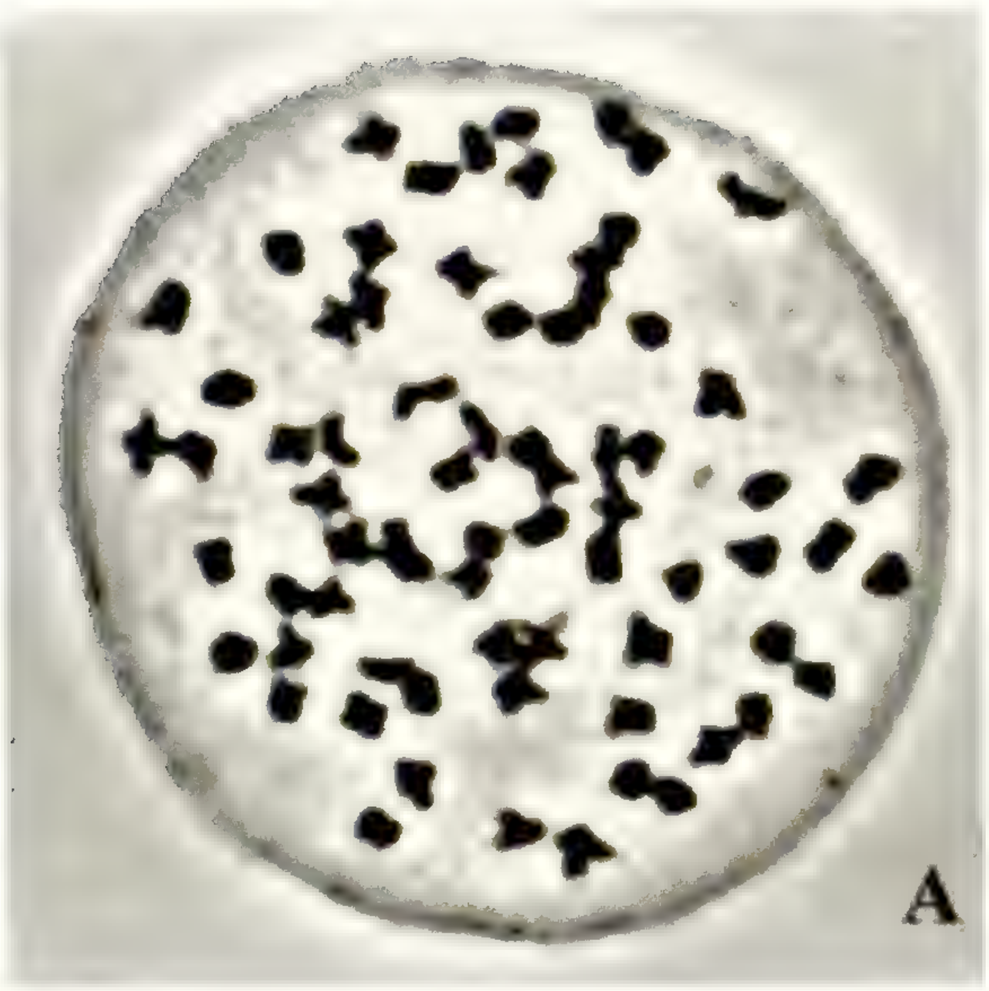
To avoid prolixity, we have combined both the results and the interpretation of the isozyme phenotypes.

Chromosomes were counted for 16 specimens collected on Gran Canaria, La Palma, and Tenerife. In addition to five tetraploid *A. aureum* ($n = 72^{II}$) plants, eleven small specimens that we could not distinguish from European *A. ceterach*, were examined. Seven of them turned out to be tetraploid ($n = 72^{II}$) and four were octoploid, having a meiotic chromosome number of $n = 144^{II}$ (Fig. 2). Meiosis in all cells examined was regular, showing only bivalents, and giving no indication about the polyploid status of the species. This agrees with Lovis' (1977) statement that most autopolyploid ferns possess diploidized meiosis (only bivalent formation), and "that the absence of multivalents is no valid evidence of allopolyploidy."

The counted samples were used as standards to determine the ploidy level of the remaining 146 specimens using a flow cytometer. Results are given in Appendix 1, 2 and 3; localities of cytologically checked material are indicated on the map of the Canarian Archipelago (Fig. 1).

In May 1995 (*RV6135*) and 1997 (*CV165–170*; *CV183–187*), we discovered tetraploid *A. ceterach* specimens on both Gran Canaria and Tenerife (see Appendix 1 and 3).

The three species (*A. aureum*, the "small tetraploid", and the octoploid) cannot always be distinguished macromorphologically, but can be identified



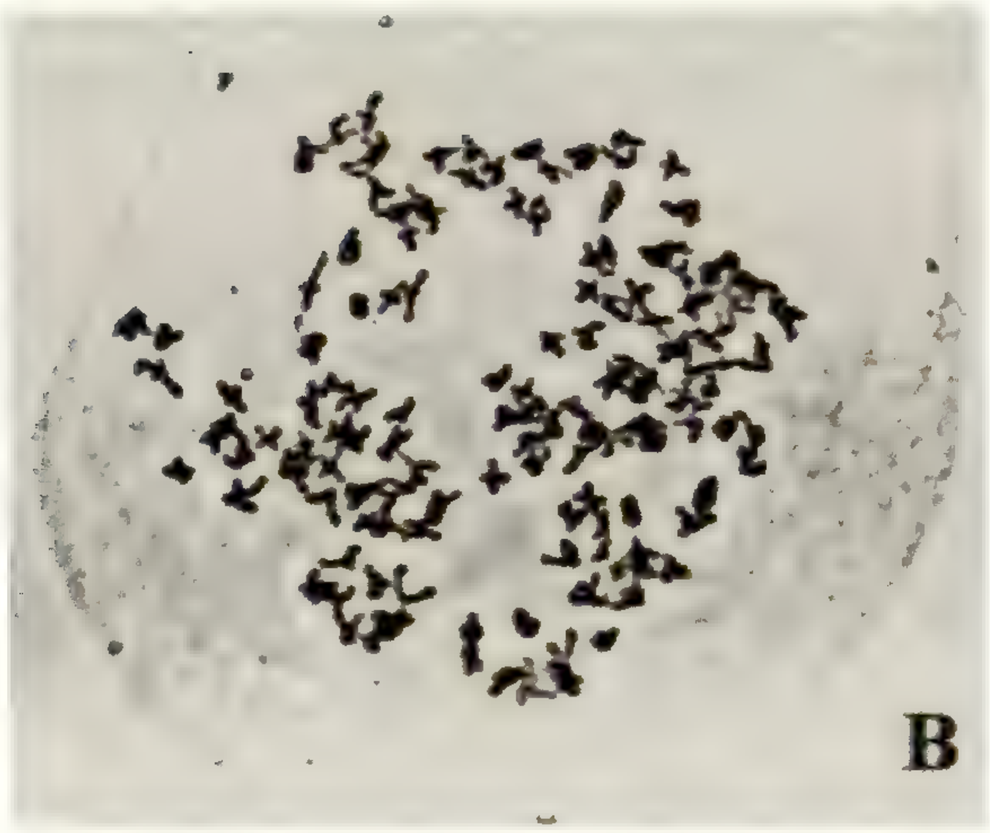


FIG. 2. Cytology showing spore mother cells in first meiotic division. A, B = photographs; A', B' = explanatory diagrams with bivalents in black.
A, A': *A. ceterach* (CV 170b), metaphase I showing n = 72^{II}. B, B': *A. octoploideum* (CV 188, holotype), cell showing n = 144^{II}. Scale bar = 10 μm. (preparations, photos and diagrams: C.V.).

by measuring exospore length (Table 2). Stomatal guard cell length can only be used to distinguish the "small tetraploid" (45 ± 3.8 μm) from the octoploid (52 ± 4.8 μm). We found no differences in perispore morphology, stomatal type, or epidermal cell pattern. Perispores have costato-cristate folds with few perforations, stomates are mesopolocytic, and epidermal cells mostly sinuous. Polyploidy factors (Viane, 1986, 1990) in *A. ceterach* are $P_{cet,\ exo} = 1.25$ (for the exospore) and $P_{cet,\ sto} = 1.16$ (for the stomates), and $P_{aur,\ exo} = 1.18$ and $P_{aur,\ sto} = 1.07$ in *A. aureum*. Using these P-values, the theoretical spore and stomate sizes calculated for the octoploid are less than 1 s.d. different from their actual means (Table 2), thus supporting the proposed ancestry (Viane, 1990).

We stress the presence of a small indusium in all taxa; it can best be observed in epidermis preparations (Viane, 1990). Our observations show that the (toothed) margin and the "cuticular lines" of the scales, are unreliable characters to

TABLE 2. Microcharacters differentiating taxa within the *A. aureum–ceterach* group on the Canary Islands. All measurements are based on cytologically checked material. Additional information about material and the number of measurements is available from the authors.

Taxon	Ploidy	Mean exospore length ± s.d.	Mean guard cell length ± s.d.
A. aurem	4x	32 ± 1.9 μm	46 ± 4.4 μm
A. ceterach	4x	39 ± 2.6 μm	45 ± 3.8 μm
A. octoploideum	8x	44 ± 3.1 μm	52 ± 4.8 μm

discriminate *A. aureum* and relatives from *A. ceterach*. All taxa have scales with more or less dentate margins, and periclinal cell walls with or without "cuticular lines". These "cuticular stripes" are folds in the periclinal cell wall (Fig. 3), and the bigger the cell the more folds seem to be present. However, *A. aureum* scales usually show numerous folds, whereas *A. ceterach* (from its entire range of distribution) paleae possess only few. In the octoploid the number of folds is usually intermediate between that in *A. aureum* and *A. ceterach*.

Isozyme analysis can be used to determine whether taxa are auto- or allopolyploid. The electrophoretic phenotype of an autopolyploid should show a subset of the isozymes present in its progenitor, assuming no mutation subsequent to the origin of the polyploid (Weeden and Wendel, 1989; Crawford, 1990; Pryer and Haufler, 1993). An allopolyploid should display fixed heterozygous (i.e., nonsegregating) banding patterns for many loci, resulting from the combination of different parental genomes (Gottlieb, 1982; Werth, *et al.* 1985b; Pryer and Haufler, 1993; Soltis and Soltis, 2000). Fixed heterozygous banding patterns differ from normal heterozygous zymograms, because the bands do not segregate among progeny and remain fixed in all specimens.

Nineteen enzyme systems were tested in a preliminary survey. The low resolution of ALD, and the smeared patterns of XDH made both unusable. IDH,

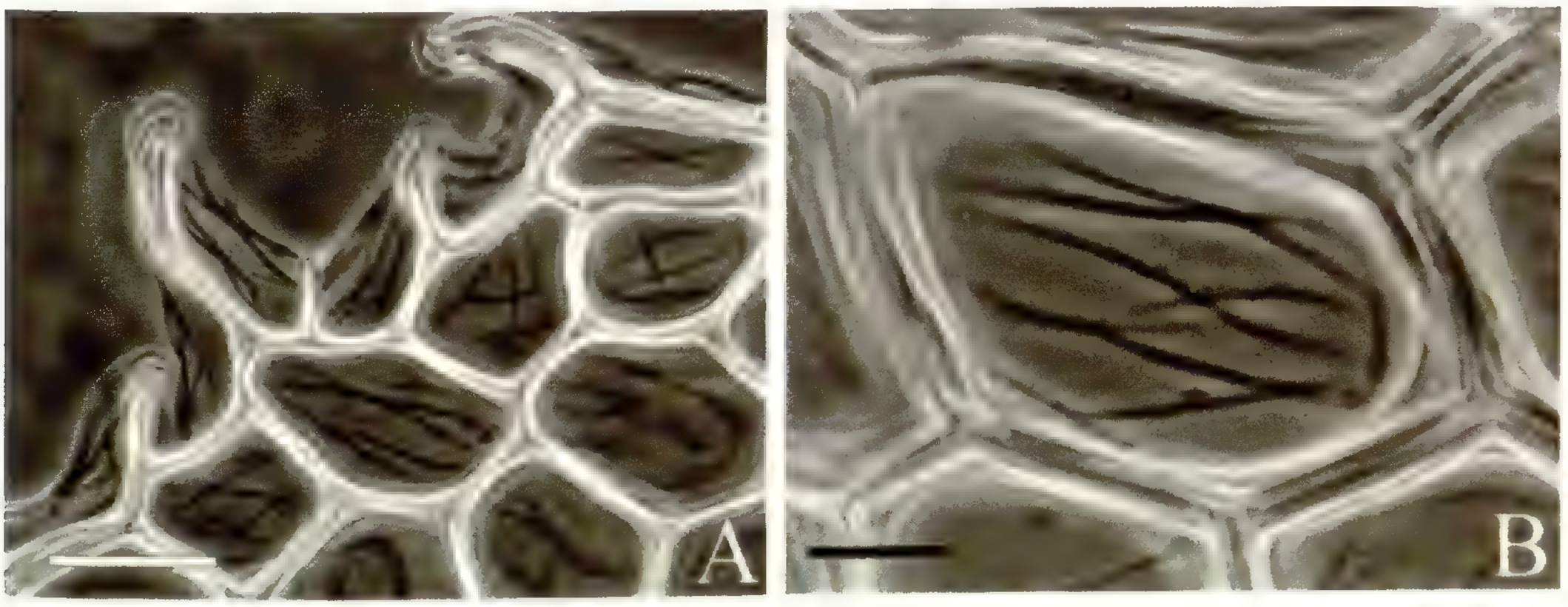


FIG. 3. Folds ("cuticular lines") in periclinal cell walls of *A. aureum* paleae. Phase contrast micrographs of laminal scales (CV 157). A: scale margin with several cells. B: single cell with folds. Bar: A = 50 μm, B = 10 μm.

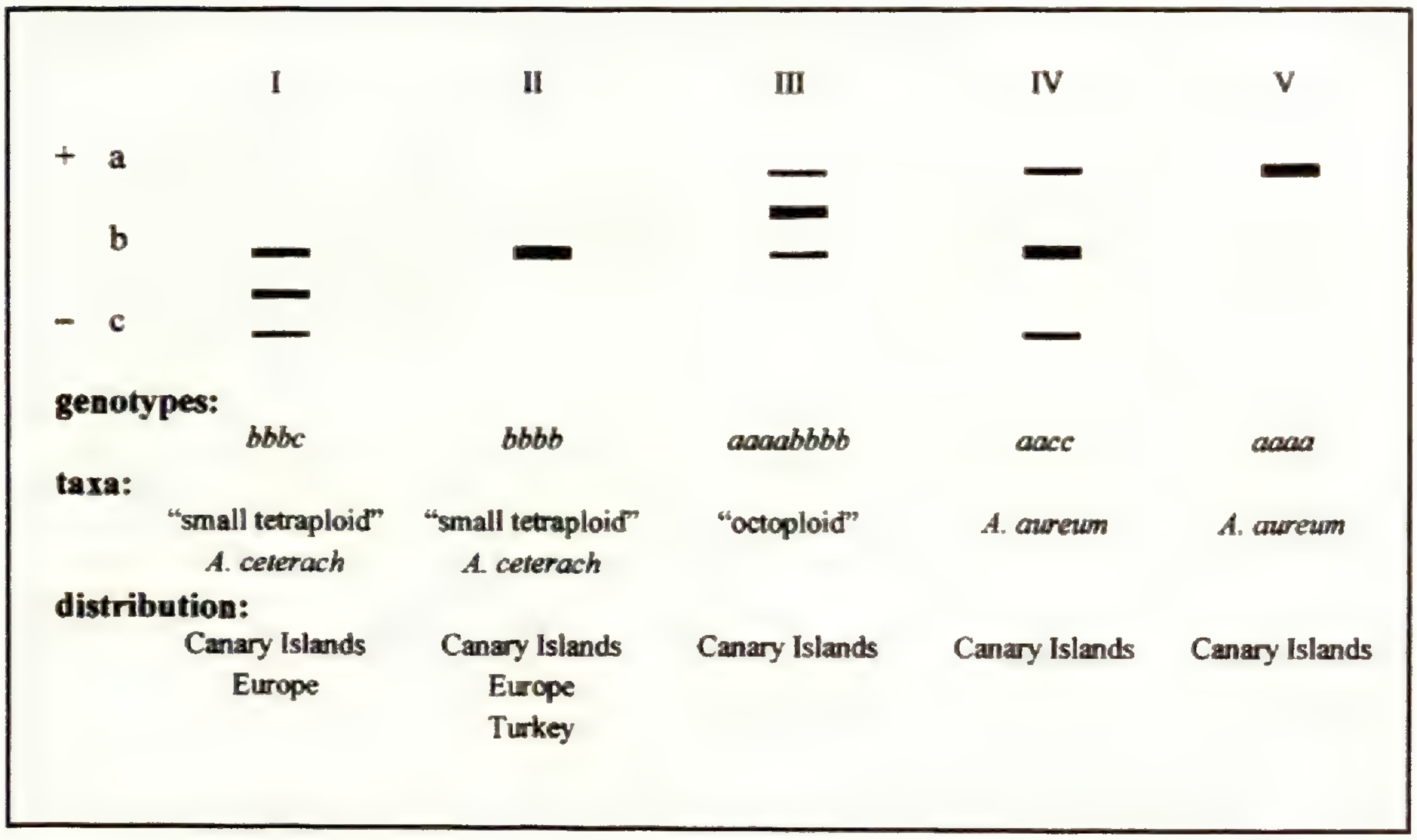


FIG. 4. Diagrams of electrophoretic AAT phenotypes, showing 3 alleles and the corresponding genotypes, as observed in the European–Canarian *Asplenium ceterach–aureum* group. Zymotype II was also found in all samples of *A. javorkeanum* (*bb*) and *A. cyprium* (*bbbbbb*) examined.

MDH, ACO, and SOD yielded unclear patterns with limited variation. G-6PD and G-3PDH gave inconsistent zymograms. Consequently, these enzyme systems were not retained.

To test our hypothesis that in addition to *A. aureum* and the octoploid, true *A. ceterach* occurs on the Canary Islands, the following four enzyme systems were suitable: AAT, SkDH, ME, and PGI. These enzyme systems yielded reproducible, well resolved banding patterns discriminating specimens representing *A. aureum*, *A. ceterach* and the allo-octoploid hybrid. These enzymes, encoded by four putative loci, were also used to get an idea of the variation of the species.

AAT or GOT: this dimeric enzyme was studied using both PAGE and SGE (system 8 of Haufler, 1985a). We observed only one activity zone, which agrees with Gastony and Darrow (1983), who proved that this single enzyme activity is chloroplastic.

Most of the specimens studied are homozygous, showing a single well-resolved AAT band (Fig. 4, zymotype II). This applies to all 50 *A. javorkeanum* specimens (Italy and Slovenia), most (102) *A. ceterach* plants (Belgium, Croatia, Cyprus, France, Italy, Slovenia, Spain, Turkey, and the United Kingdom), 23 "small tetraploids" from the Canary Islands, and all (44) *A. cyprium* samples tested (Van den heede *et al.* 2002). Ten *A. ceterach* individuals from Croatia, France, Italy, and Slovenia, and two "small tetraploids" from the Canary Islands, showed the rarer three-banded zymotype I with skewed staining intensities, interpretable as heterozygous for a dimer.

Corresponding genotypes are *bb*, *bbbb*, or *bbbbbb* for zymotype II of the homozygous (diploid to hexaploid) plants, versus *bbbc* for zymotype I of the heterozygous tetraploid specimens.

Identical banding patterns in the Canarian "small tetraploid" and in European *A. ceterach* plants indicate that true *A. ceterach* is growing on the Canary Islands. Because neither European nor Canarian samples reveal unique electrophoretic phenotypes, the Canarian populations do not seem to be genetically isolated. Though more sampling of *A. javorkeanum* is needed, these preliminary results (Fig. 4, zymotype II) seem to confirm the autotetraploidy of *A. ceterach*.

The *a* allele (Fig. 4, zymotype V) can be used as a "marker" allele characterizing *A. aureum*. Some plants, showing a single band corresponding to genotype *aaaa*, are homozygous, whereas others, with a balanced three-banded zymogram corresponding to genotype *aacc*, are heterozygous. To explain zymotypes I and IV, an extra genotype *cc* is postulated and expected in *A. javorkeanum*, which was studied only on the basis of Italian and Slovenian material.

All 54 octoploid specimens (from Gran Canaria, La Palma, and Tenerife) show a monomorphic, presumably fixed heterozygous banding pattern of genotype *aaaabbbb* (Fig. 4, zymotype III), which we postulate to be derived from a combination of zymotypes II and V (Fig. 4). This would agree with the suggestions of Reichstein (1984) and Viane and Reichstein (1992), that the Canarian octoploid is an allo-octoploid, which originated either by chromosome doubling in an unknown tetraploid hybrid between *A. aureum* (with zymotype V) and *A. ceterach* (with zymotype II), or via unreduced gametes of each species (Fig. 9). The fact that, in all the octoploids (from 15 different localities) only one AAT zymotype was detected can be explained by the preponderance of the "small tetraploid" with zymotype II. It may also reflect incomplete sampling of the variation present in the octoploid.

SkDH: resolution for this monomeric enzyme was superior on SGE (system 2 of Weeden and Wendel, 1989). In our study, the enzyme was represented by a single locus, which agrees with Gastony and Darrow (1983). As expected for a monomeric enzyme, homozygotes had a typical one-banded pattern whereas heterozygotes showed two or more bands.

We detected four alleles in the European–Macaronesian *Asplenium ceterach–aureum* group. Two of these, *a* and *b*, were observed in *A. aureum*, whereas *c* and *d* characterized the "*A. ceterach*" group. Although SkDH was polymorphic in *A. ceterach* (Fig. 5), only zymotype V (*cccd*) was found on the Canary Islands. This two-banded pattern with unequal staining intensities forms also part of zymotype VI found for all 54 octoploid specimens. Thus the octoploid is monomorphic and presumably heterozygous for this locus, showing a four-banded zymogram corresponding to genotype *aabbcccd*. The *a* and *b* alleles are unique "marker" alleles for *A. aureum*, one of the progenitors of the octoploid. This monomorphic pattern showing presumed fixed heterozygosity seems to confirm the putative allo-octoploid origin of this species (Reichstein, 1984; Viane and Reichstein, 1992). The *cccd* SkDH

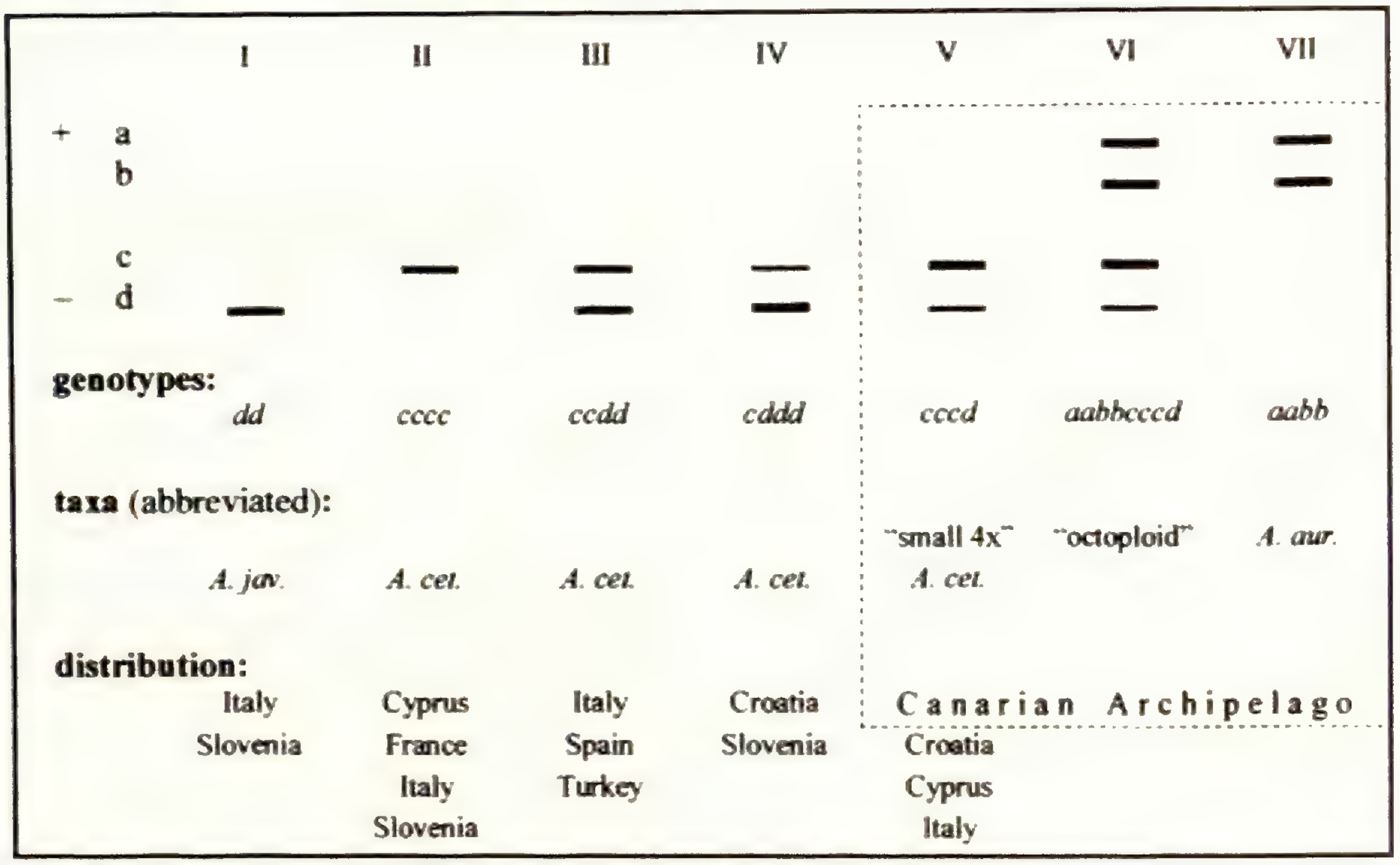


FIG. 5. Diagrams of electrophoretic SkDH phenotypes, with corresponding genotypes, as observed in the European–Canarian *Asplenium ceterach–aureum* group. Zymotype II was also found in some *A. javorkeanum* (genotype: *cc*); zymotype V was present in all *A. cyprium* (genotype: *ccccdd*) samples checked. The Canarian small tetraploid is abbreviated as: "small 4x."

genotype (zymotype V) of the "small tetraploids" is not limited to the Canaries, but was also found in *A. ceterach* from Croatia, Cyprus, and Italy. These results again both prove the occurrence of *A. ceterach* on the Canary Islands, and the fact that the populations in this Archipelago are not genetically isolated. Three additional zymotypes were detected in continental *A. ceterach*: a single-banded (*cccc*), a balanced two-banded (*ccdd*), and an unbalanced two-banded pattern (*cddd*). The presence of the unbalanced patterns (*cccd, cddd*) in tetraploid *A. ceterach* at *Skdh* can be explained by tetrasomic inheritance (see discussion). Diploid *A. javorkeanum* from Italy and Slovenia showed a single-banded pattern of either genotype *cc* (zymotype II) or *dd* (zymotype I).

ME: this tetrameric enzyme was studied only by PAGE. The single enzyme activity visible was shown to be cytosolic by Gottlieb (1982), Gastony and Darrow (1983), and Soltis (1986).

ME was monomorphic in each of the three species, and thus can be used to distinguish them from each other (Fig. 6). All *A. ceterach* specimens (Europe) and "small tetraploids" (Canary Islands) were heterozygous showing an identical five-banded zymogram, typical for a tetrameric enzyme controlled by one locus with two alleles, *a* and *d*. Heterozygous *A. aureum* was characterized by a five-banded pattern controlled by the same locus, but with two different alleles, *b* and *c*, and corresponding to genotype *bbcc*. All octoploid plants

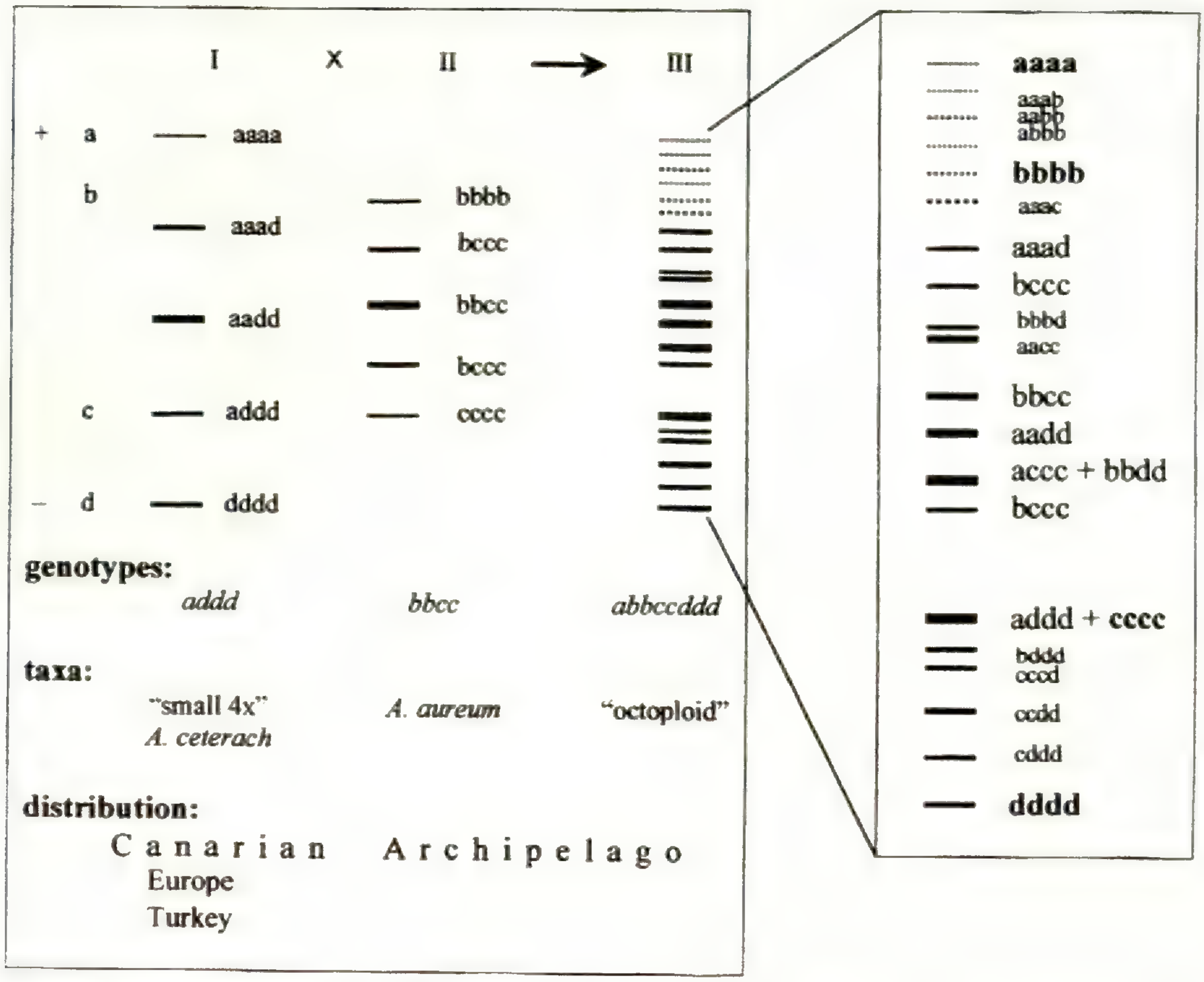


FIG. 6. Diagrams explaining the ME zymotypes showing 4 alleles, with corresponding genotypes, as observed in the European–Canarian *Asplenium ceterach–aureum* group. For each band four letters represent the association of subunits (coded by alleles) joined to form this tetrameric enzyme. The homotetramers in the "hybrid" pattern are in boldface. Zymotype III confirms the allopolyploid status of the octoploid. Dotted lines indicate very faint bands. The Canarian small tetraploid is abbreviated as "small 4x."

showed a complex zymogram, and conform to the expected hybrid phenotype resulting from the cross between *A. ceterach* and *A. aureum*. The "hybrid" had the four parental alleles, and since ME is a tetramer, each of the six pairs of alleles ($a \times b$, $a \times c$, $a \times d$, $b \times c$, $b \times d$, $c \times d$) formed three heterotetramers of intermediate mobility. Theoretically this results in a 22-banded pattern (4 homotetramers plus $6 \times 3 = 18$ heterotetramers, makes 22 bands), but because twice two bands have the same mobility, a maximum of 20 bands was visible (Fig. 6). The monomorphic and presumably fixed banding pattern of the "hybrid" zymogram is in agreement with the putative allopolyploid origin of the octoploid (Reichstein, 1984; Viane and Reichstein, 1992).

PGI: this dimeric enzyme was studied by both PAGE and SGE. Because the resolution was much better with SGE, all results shown were obtained using starch gel electrophoresis (system 6 of Soltis *et al.*, 1983).

Two loci were present: *Pgi-1*, most probably chloroplastic, and *Pgi-2*, cyto-

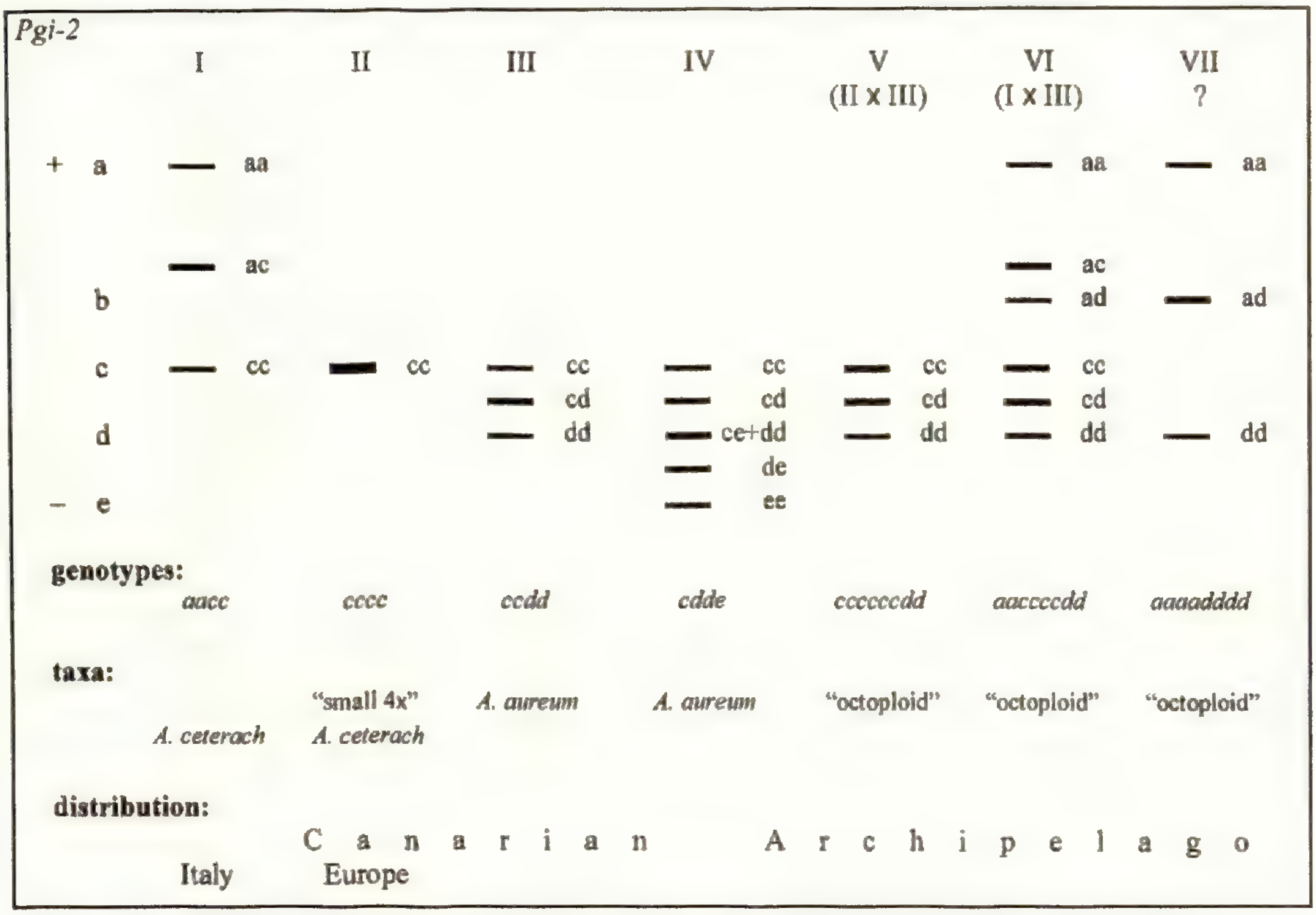


FIG. 7. Diagrams of electrophoretic *Pgi-2* phenotypes, showing 5 alleles, with corresponding genotypes and their distribution, as observed in the European–Canarian *Asplenium ceterach–aureum* group. Zymotype II was also found in most *A. javorkeanum* (*cc*) samples. Each band is indicated by two letters representing the association of subunits, joined to form this dimeric enzyme. The Canarian small tetraploid is abbreviated as "small 4x."

solic (Gastony and Darrow, 1983; Soltis, 1986). Consistent with observations on other ferns (Gastony and Gottlieb, 1985; Werth, 1991; Haufler *et al.*, 1995; Hauk and Haufler, 1999), resolution of the more anodal locus *Pgi-1* was inferior to that of *Pgi-2*. Because *Pgi-1* appears invariant across all taxa, it will not be discussed.

Among the European and Macaronesian samples studied, five allozymes were observed at *Pgi-2* (Fig. 7). Although the continental *A. ceterach*, with its six different zymotypes, was highly polymorphic for this locus (Van den heede *et al.*, 2002), only a single banding pattern was detected for the 22 "small tetraploids" from the Canary Islands, corresponding to genotype *cccc*. This widely distributed zymotype was also found in *A. ceterach* specimens from Belgium, Croatia, France, Italy, Slovenia, Spain, and the United Kingdom. We obtained two electrophoretic phenotypes for the 28 *A. aureum* plants, with corresponding genotypes *ccdd* (25 specimens) and *cdde* (3 specimens). The octoploid was the most variable taxon in the Canarian Archipelago, showing three different zymotypes translated into genotypes (Fig. 7) *ccccccdd* (zymotype V), *aacccccdd* (zymotype VI), and *aaaadddd* (zymotype VII). Zymotype V (found only on La Palma) most probably resulted from hybridization

TABLE 3. Genbank accession numbers for *trnL-trnF* nucleotide sequences of newly sequenced *Asplenium* specimens. CV and TR are abbreviations for Caroline Van den heede and Tadeus Reichstein respectively. Localities are given in Appendix 4.

Species	Voucher number	Data of collection	GenBank accession number
A. aureum	*CV164*	25 May 1997	AY160993
	CV670	12 Jan. 1999	AY160994
	CV712	3 Apr. 1999	AY160995
A. ceterach	*CV187*	27 May 1997	AY162333
	CV225	11 June 1997	AY162334
	CV494	17 Aug. 1998	AY162335
A. cyprium	*CV249*	12 June 1997	AY162337
A. dalhousiae	*CV318*	13 Jan. 1998	AY161000
	TR7634	27 Aug. 1990	AY161001
A. javorkeanum	*CV14*	24 July 1996	AY162330
	CV85	30 Aug. 1996	AY162331
A. lolegnamense	*CV985*	29 May 2000	AY160998
	CV993	1 June 2000	AY160999
A. octoploideum	*CV709*	2 Apr. 1999	AY161003

between a "small tetraploid" with genotype *cccc* and an *A. aureum* with genotype *ccdd*, which are both abundantly present on the Canaries, followed by chromosome doubling, or via unreduced gametes of each species. Zymotype I, though presently known only from Italy, can be used to explain zymotype VI, which was found only on La Palma. Octoploids with this genotype (*aacccccdd*), expressed three homodimeric bands (Fig. 7, aa, cc, dd) plus three heterodimeric bands (ac, ad, cd). More sampling is desirable and might detect other genotypes such as *aacc* in the "small tetraploid," as well as *dddd* needed to explain zymotype VII from Gran Canaria and Tenerife. *Pgi-2* suggests that the formation of the allo-octoploid happened at least three times.

Because plastid DNA is uniparentally inherited, it discloses only the maternal lineage (Stein and Barrington, 1990; Gastony and Yatskievych, 1992). GenBank accession numbers for *trnL-trnF* nucleotide sequences of newly sequenced specimens are listed in Table 3. Analysis of the plastid *trnL-trnF* intergenic spacer sequences resulted in the clustering of the "small tetraploid" from Tenerife (*CV187*), *A. ceterach* from Italy and Cyprus, and *A. cyprium*, with their diploid ancestor *A. javorkeanum* (Fig. 8). We found no chloroplast variation (with the exception of *CV494*) between specimens sampled from the Mediterranean (Cyprus, Italy, Slovenia) and Tenerife. *Asplenium javorkeanum*, *A. ceterach*, and *A. cyprium* form a cluster of their own, different from the "*A. aureum* clade," which includes all the *A. aureum* specimens, *A. lolegnamense*, and the octoploid (*CV709*) from the Canaries. Identical groups are obtained by analysing *rbcL* gene sequences. The position of *A. lolegnamense* and the octoploid, in the plastid trees, suggests that *A. aureum* acted as the maternal parent in the formation of the specimens used. These molecular data independently prove that in addition to an octoploid species, true *A. ceterach*

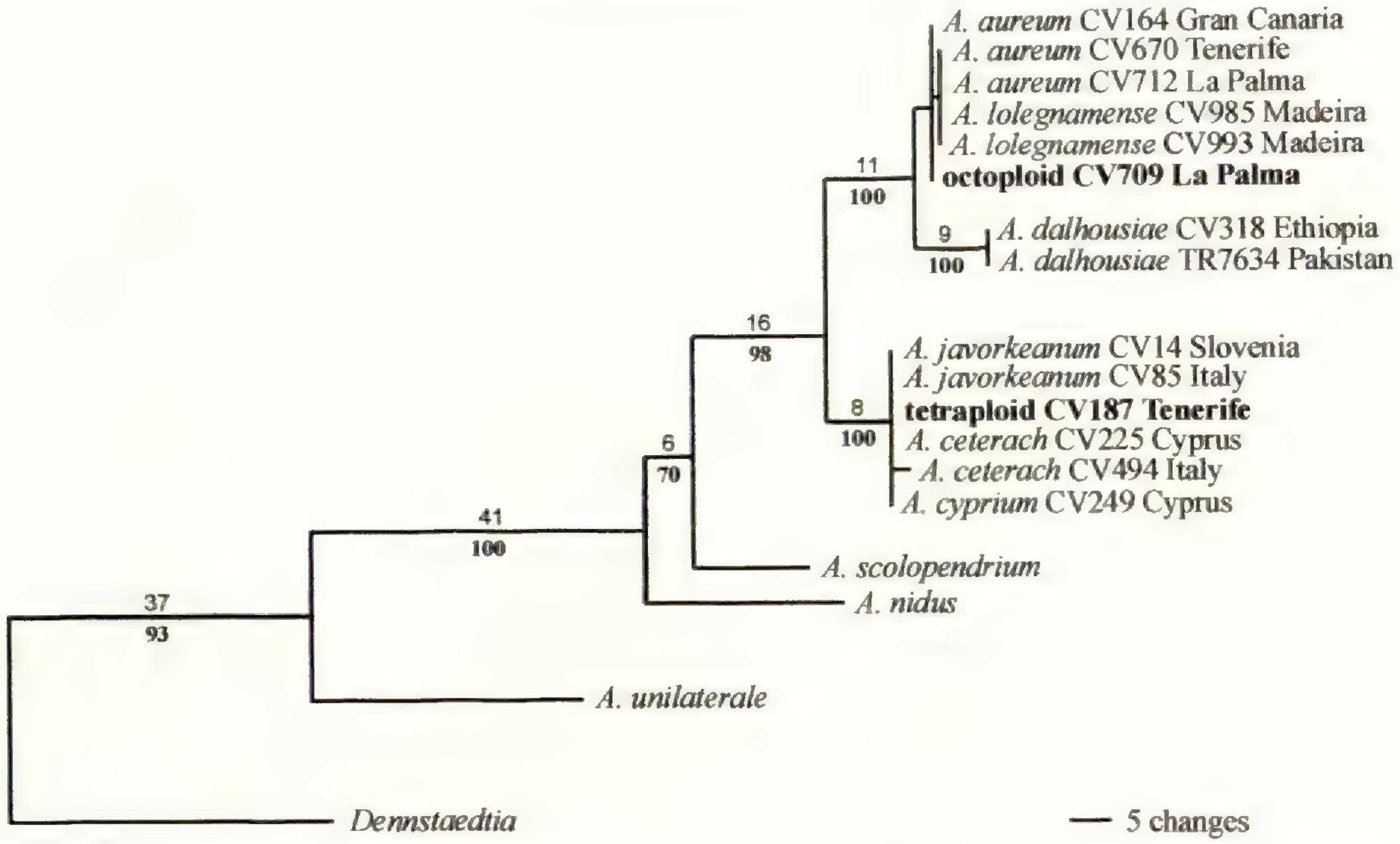


FIG. 8. Tree randomly selected from the 73 shortest trees of European–Canarian *Asplenium ceterach–aureum* taxa and *A. dalhousiae*, resulting from parsimony analysis of our 14 *trnL-trnF* intergenic spacer sequences (Table 3) and 4 *trnL-trnF* sequences of other species available in GenBank; length = 227 steps, CI = 0.92, and RI = 0.91. Based on *rbcL* evidence (Hasebe et al., 1995; Pryer et al., 1995), the sequence of the more distantly related *Dennstaedtia* was specified as outgroup. Fitch branch lengths (ACCTRAN optimized) are shown above and bootstrap percentages (1000 replicates) below the branches. Other sequencing results are described extensively in Van den heede et al. (2003).

(= the "small tetraploid") is growing on the Canary Islands. Other sequencing results are described extensively in Van den heede *et al.* (2003).

Because Gran Canaria, La Palma, and Tenerife are of volcanic origin, these epilithic ferns mainly grow on rocks of basaltic types, like phonolites, rhyolites, trachytes and olivine basalts (Page, 1979). *Asplenium aureum* prefers moister, shady habitats at lower altitudes, whereas the "small tetraploid" and octoploid plants share more exposed, drier habitats. However, on both Gran Canaria and Tenerife, only single localities were found where "small tetraploids" and octoploids grew together (loc. 2 and 19; see Appendix 1 and 3). Though forty-five specimens from nine different localities on La Palma were cytologically checked (see Appendix 2 and Van den heede and Viane, unpublished data), we could not detect any "small tetraploid" specimen. We intensively looked for it in the field, especially in the higher regions of La Palma. Whereas we discovered "small tetraploids" between 1500 and 1900 m altitude on Gran Canaria and Tenerife, the highest altitude we found ferns of the *Ceterach* group on La Palma was near 1200 m.

We found *A. aureum* between 300 and 1000 m altitude in valleys and sheltered ravines ("barrancos") with remnants of (degraded) evergreen laurel

forest dominated by broad-leaved trees: *Laurus azorica* (Seub.) Franco, *Persea indica* (L.) Spreng., *Ocotea foetens* (Aiton) Berthel., *Apollonias barbujana* (Cav.) Bornm., *Ilex canariensis* Poir., *Ilex platyphylla* Webb and Berthel., and *Arbutus canariensis* Vieill. (Bramwell and Bramwell, 1974). *Asplenium aureum* usually grows in humus-rich soils, often together with *Adiantum capillus-veneris* L., *A. reniforme* L., *Anogramma leptophylla* (L.) Link, *Asplenium aethiopicum* (Burm.f) Bech., *A. hemionitis* L., *Cheilanthes pulchella* Bory ex Willd., *Davallia canariensis* (L.) Sm., *Polypodium cambricum* L. ssp. *macaronesicum* (A.E. Bobrov) Fraser-Jenk., and *Selaginella denticulata* (L.) Spring.

Asplenium ceterach and *A. octoploideum* were found in the natural pine forests ("Pinar") at 900–2000 m on Tenerife, and at 1200–1600 m on Gran Canaria. The octoploid was observed on La Palma at 700–1200 m. The open savannah-like vegetation is dominated by *Pinus canariensis* C. Sm. and a few shrubs, such as *Adenocarpus foliolosus* (Aiton) DC., *Cistus symphytifolius* Lam., *Daphne gnidium* L., *Micromeria* species, and *Rumex lunaria* L. (Bramwell and Bramwell, 1974). *Asplenium ceterach* and the octoploid grow in rock fissures, often together with *Monanthes laxiflora* (DC.) Bolle, *Aeonium* species, *Asplenium aethiopicum*, *A. trichomanes* L., *Anogramma leptophylla*, *Cheilanthes guanchica* Bolle, *C. pulchella*, *Cosentinia vellea* (Aiton) Tod., *Notholaena marantae* (L.) Desv. subsp. *subcordata* (Cav.) G. Kunkel, and *Polypodium cambricum* ssp. *macaronesicum*. Where *A. ceterach* and *A. octoploideum* grow together abundantly, we discovered their sterile hexaploid hybrid, *A.* ×*chasmophilum* Van den heede and Viane (Van den heede and Viane, 2002)

Discussion

In combination with morphological, cytological, and biogeographical data, isozyme markers can determine whether taxa are auto- or allopolyploid (Crawford, 1985; Haufler, 1985b; Bryan and Soltis, 1987; Weeden and Wendel, 1989; Crawford, 1990; Gastony, 1990; Pryer and Haufler, 1993). Electrophoretic analysis of isozymes is an ideal way to investigate the origin of allopolyploid taxa because parental loci are expressed as stable marker bands in the progeny (Haufler, 1985b; Werth *et al.*, 1985b; Gastony, 1986). The potential of isozyme data to clarify relationships in fern complexes is dependent upon the degree of differentiation among the ancestral genomes.

In the present study, four loci (*Aat, Skdh, Me*, and *Pgi-2*) showing a unique set of bands characterizing *A. aureum* and different from the banding patterns found in European *A. ceterach*, proved adequate to disentangle the "*Ceterach*" complex in the Canarian Archipelago.

All zymograms present in the Canarian "small tetraploid", were also observed in *A. ceterach*, and confirm that true *A. ceterach* is growing on the Canary Islands. Moreover, this suggests an occasional spore flow from Europe towards the Canaries. A flow in the opposite direction is less likely because the western islands are dominated by the northeast trade wind system. Conse-

quently, the Canarian *A. ceterach* population cannot be considered genetically isolated. No local zymotypes seem to have originated in this taxon in the Canary Islands, contrary to the situation on Cyprus, which is much older than the Canary Islands (Van den heede *et al.*, 2002). For example, the unique *Tpi-2* zymogram, present in all *A. ceterach* and *A. cyprium* specimens from Cyprus, suggests the local origin of the Cypriot taxa (Van den heede *et al.*, 2002).

All four loci (*Aat, Skdh, Me,* and *Pgi-2*) of the Canarian octoploid show monomorphic heterozygosity for a combination of the patterns seen in *A. ceterach* and *A. aureum*. Our allozyme data confirm the allo-octoploid nature of this species, which most probably originated by chromosome doubling in a tetraploid hybrid between *A. aureum* and *A. ceterach* (Viane and Reichstein, 1992). Theoretically, though less parsimoniously, the formation of this taxon could also happen directly via the union of unreduced (4x) gametes (on gametophytes resulting from unreduced spores) of both species.

All allozymes observed in the allo-octoploid were electrophoretically identical to those found in the parental tetraploids. However, in some octoploid samples from Gran Canaria and Tenerife, *Pgi-2* expressed a zymotype (corresponding to genotype *aaaadddd*) resulting from the combination of two undetected genotypes (*aaaa*) in *A. ceterach* and (*dddd*) in *A. aureum*. The occurrence of this putative "orphan" genotype may reflect incomplete sampling of the variation present in the tetraploids, or alternatively these genotypes may no longer be present in extant *A. ceterach* and *A. aureum* specimens.

The variation in the allo-octoploid seems to be related to the mono- or polymorphism (and its abundance) in the parental tetraploids. Thus, at the two loci (*Skdh* and *Me*) showing a single octoploid genotype, only one genotype was observed in each of the Canarian parents. At *Aat* two different genotypes were found for the Canarian *A. ceterach*, though only a single zymotype was detected for the octoploid. However, the *A. ceterach* genotype not detected in any octoploid, was found in only ca. 10% of the population. On the other hand, this may also be the result of limited sampling of the variation present in the octoploid.

The allo-octoploid species showed three different isozyme profiles at *Pgi-2*, a locus that is polymorphic in its tetraploid progenitors, indicating that the octoploid probably originated at least three times. According to Werth *et al.* (1985a) such patterns of variation in allopolyploids are almost certainly the result of repeated allopolyploidizations involving pairs of different genotypes. Thus, each of the octoploid zymotypes may have arisen from a separate hybridization event. The present observations, demonstrating multiple origins of allopolyploids, are similar to those of Werth *et al.* (1985a, b) for *Asplenium*, Soltis et al. (1987) for *Polystichum*, and Ranker et al. (1989) for *Hemionitis*. Recurrent origins of the allo-octoploid species implicate a repeated gene flow from tetraploids to octoploids, and mean a continued gain of genetic diversity by the allopolyploid.

Our electrophoretic data also provide evidence for the operation of tetrasomic inheritance in natural populations of autotetraploid (Rasbach *et*

al., 1987) *A. ceterach*. At *Skdh*, for which only two allozymes were observed, three types of heterozygotes were present: balanced heterozygotes (*ccdd*) and two types of unbalanced heterozygotes (*cccd, cddd*). The presence of these three types in tetraploid *A. ceterach* at *Skdh* is suggestive of the three possible classes of heterozygotes expected in an autotetraploid at a locus having two alleles (Weeden and Wendel 1989). Unbalanced staining activities indicate multiple doses of individual alleles. Tetrasomic inheritance with chromatidal segregation explains the arrays of homozygous, balanced heterozygous, and unbalanced heterozygous banding patterns observed in *A. ceterach* (Weeden and Wendel 1989). Tetrasomic inheritance implies that a chromosome can pair with any of its three homologous chromosomes (e.g., Soltis and Rieseberg, 1986; Weeden and Wendel, 1989; Crawford, 1990), and that there is apparently no strict preferential chromosome pairing. Consequently, the present isozyme analysis confirms the autotetraploid status of *A. ceterach*, which was cytologically proven by Rasbach et al. (1987). The fact that isozyme studies point to tetrasomic inheritance and that we found only bivalents during meiosis in autotetraploid *A. ceterach*, suggests that both processes are controlled by different (sets of) genes. Similar unbalanced patterns found in allotetraploid ferns have been explained also by segregating intralocus heterozygosity and fixed interlocus heterozygosity (Gastony, 1990).

We were able to prove, by isozyme and plastid DNA analysis, that in addition to *A. aureum* and the octoploid, true *A. ceterach* occurs on Gran Canaria and Tenerife. A combination of morphological and cytological analysis leads to correct determination, but even the exospore length alone allows reliable identification of the three Canarian species: *A. aureum* (32 ± 1.9 μm), *A. ceterach* (39 ± 2.6 μm), and the octoploid (44 ± 3.1 μm).

As mentioned in the introduction, Benl and Kunkel (1967) published *C. aureum* var. *parvifolium* without cytological investigation. Plants collected in 1967 by T. Reichstein and G. Kunkel were found to be octoploid, leading T. Reichstein and other European pteridologists to attribute octoploid status to *A. parvifolium* (including all small Canarian "*Ceterach*" specimens), but without having checked the holotype.

We repeatedly visited the type locality of *A. parvifolium*, the Pinar above Vilaflor (Tenerife), and found several taxa (see Appendix 3) growing together. As soon as we were convinced that two kinds of "small *Ceterach*" species were growing at the locus classicus (and on Gran Canaria), we decided to study the holotype (*Benl s.n.*, 26/12/1966, M) of *C. aureum* var. *parvifolium*. This holotype consists of one single plant. We studied its microcharacters (see also Table 4) and found a mean exospore length (38 ± 2.8 μm) and very few folds ("cuticular lines") in the scales characteristic for true *A. ceterach*. The values for the exospore and stomate length (38 ± 3.7 μm) prove that the holotype was not an octoploid, but a tetraploid plant! Consequently, *C. aureum* var. *parvifolium* Benl and G.Kunkel and *A. parvifolium* are synonyms of *A. ceterach*, and the octoploid had no correct name and was described as *A. octoploideum* (Van den heede and Viane, 2002). *Asplenium octoploideum* is morphologically intermediate between *A. aureum* and *A. ceterach*, from which

TABLE 4. Comparison of mean exospore length (LEXO) of various types to that of cytologically checked vouchers (see Table 2).

Taxon	Voucher, status, and herbarium	LEXO ± s.d. (types)	LEXO ± s.d. (cytol. checked)
A. aureum	*Broussonet s. n.*, iso-:P	31 ± 1.6 μm	32 ± 1.9 μm
A. ceterach	*Hort. Cliff. Aspl. 4*, lecto-: BM	39 ± 2.8 μm	39 ± 2.6 μm
A. parvifolium	*Benl s. n.*, holo-: M	38 ± 2.8 μm	
A. octoploideum	*CV 188*, holo-: GENT	42 ± 3.4 μm	44 ± 3.1 μm

it can be distinguished by its different mean exospore length (44 μm) and mean stomate length (52 μm), and its octoploid chromosome number $n = 144^{II}$ (Fig. 2B + B'). It is endemic to the Canarian Archipelago but presently confirmed only (cytology) for Gran Canaria, La Palma, and Tenerife, and to be expected on La Gomera and El Hierro. In addition to the holotype from Gran Canaria [lava field near Cueva Corcho, in fissures of volcanic rocks, 1350 m alt, 28th May 1997, leg. *Van den heede and Viane CV 188* (Holo-: GENT, iso-: personal herbarium of Viane and Van den heede)], the following collections (paratypes) were also made (for localities see Appendix 1, 2, and 3): *CV 171, CV 172, CV 173, CV 175, CV 176, CV 177, CV 179, CV 672A+B, CV 674, CV 686, CV 687, CV 695, CV 708, CV 709, CV 715, CV 716, CV 717, CV 718, CV 719A+B, CV 720, CV 721, CV 723, CV 724, CV 725, CV 726, CV 727, CV 729, CV 730A+B, CV 731, CV 732, CV 733, CV 734, CV 740, CV 741, CV 742, CV 743, CV 744, CV 745, CV 746, CV 747, CV 748, CV 749, CV 750, CV 751A+B, CV 752, CV 754A+B+C, WB 22/93.*

Many herbarium specimens still need to be inspected before the ranges of the "small Canarian *Ceterach*" taxa can be established. Literature references and information on herbarium labels are often unreliable, e.g., *Bornmüller 3094* (P), labeled as *C. officinarum* f. *typica* (*cellulis palearum non striatis*!) collected on Gran Canaria, has a mean exospore length of 44 ± 3.0 μm and numerous folds in the scale cells: it is without any doubt an octoploid.

As far as is known, the octoploid is endemic to the Canary Islands, but because Madeira could also harbor this species (climatologically and topographically), we studied 30 specimens from five Madeiran localities. However, all of them turned out to be hexaploid and were identified as *A. lolegnamense*. Both in the Madeiran and the Canarian Archipelago the northeast trade wind prevails during the year. This phenomenon may help to explain the restricted range of several Macaronesian taxa, because most propagules fall into the Atlantic Ocean. Even when spores occasionally reach the African continent, the Western Sahara and Mauritania offer no appropriate habitats for these ferns, because of their ultra dry climate. The derivation we hypothesize for the allo-octoploid species is presented in Fig. 9.

It is generally accepted (e.g., Burchard, 1929; Lems, 1960; Page, 1973; Bramwell and Bramwell, 1974; Page, 1977, 1979) that many of the Canarian ferns are endemic relicts of the Tertiary fern flora that existed in southern Europe during the Miocene and Pliocene. These ferns form an important part of the original vegetation of the Canary Islands, especially of the evergreen

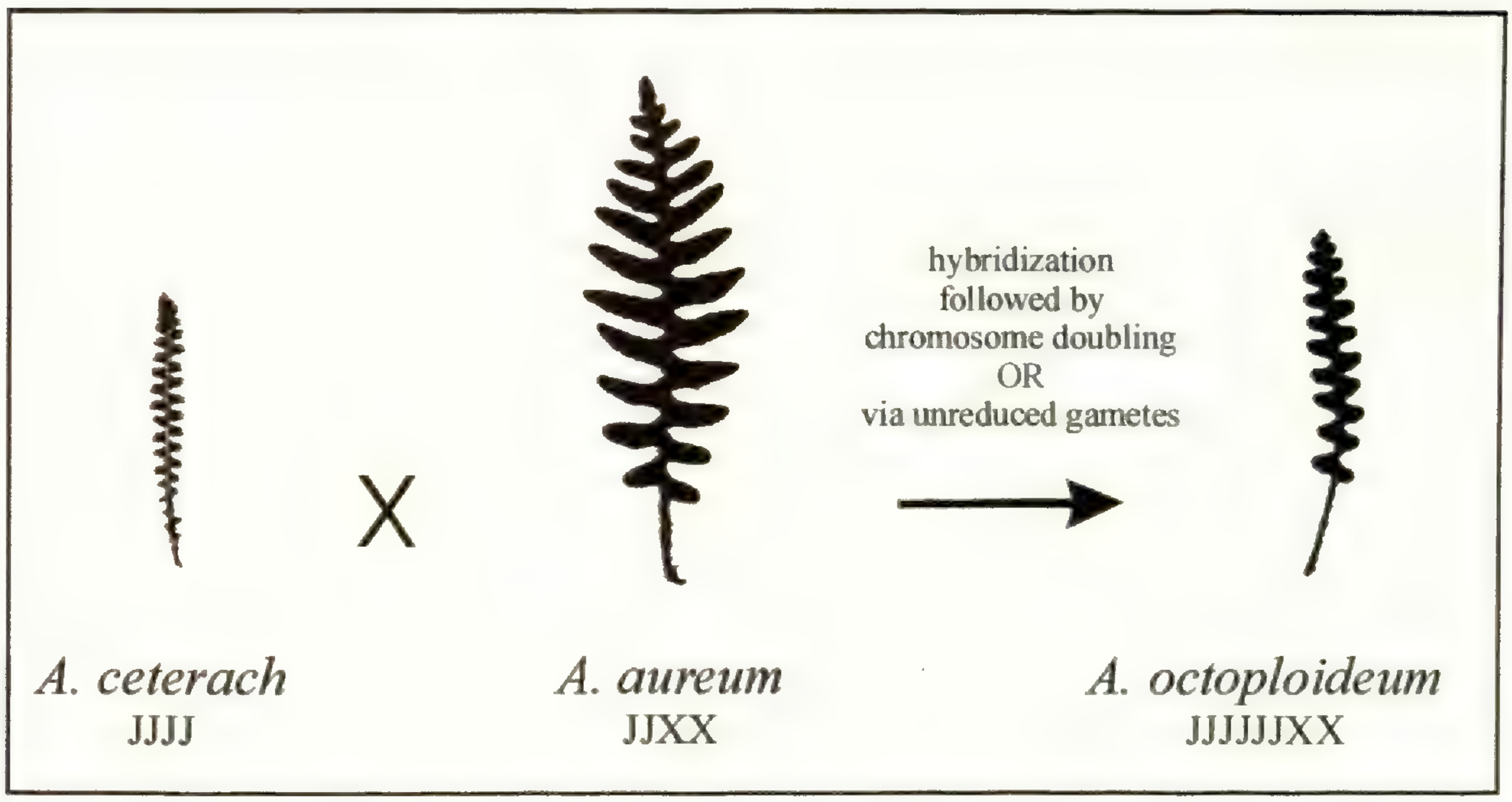


FIG. 9. Scheme of relationships explaining the origin of the Canarian *Asplenium octoploideum* based on (micro)morphological, cytological and molecular data. Each capital represents one set of 36 ancestral chromosomes. The JJ genome represents *A. javorkeanum*. Because molecular studies (Van den heede et al., 2003) suggest that *A. aureum* is an allotetraploid, involving *A. javorkeanum* (JJ) and "*A. semi-aureum*" (XX, unknown) as ancestors, its genome formula is given as JJXX.

forests (Page, 1977). Unfortunately, these habitats, if not totally destroyed today, are greatly endangered by modern tourism (building, water supply). Several mountain areas need further research, and new species and hybrids await description. Undoubtedly, this Tertiary (fern) flora forms an irreplaceable genetic resource that should be conserved.

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APPENDIX 1. Vouchers from Gran Canaria, with corresponding taxa, locality numbers, and chromosome numbers. CV is the abbreviation for Caroline Van de heede. The description of the localities and their number is given in Material and Methods. For samples with chromosomes counted, the number of bivalents is given. Counted specimens served as standards to determine the ploidy level (indicated by 4x and 8x) by flow cytometry. All specimens were used for isozyme analysis.

Voucher number	Taxon	Locality	Date of collection	Meiotic chromosome number (n), or ploidy
CV 157	*A. aureum*	1	25 May 1997	72^{II}
CV 158	*A. aureum*	1	25 May 1997	4x
CV 159	*A. aureum*	1	25 May 1997	4x
CV 160	*A. aureum*	1	25 May 1997	72^{II}
CV 161	*A. aureum*	1	25 May 1997	72^{II}
CV 162	*A. aureum*	1	25 May 1997	4x
CV 163	*A. aureum*	1	25 May 1997	4x
CV 164	*A. aureum*	1	25 May 1997	72^{II}
CV 165	*A. ceterach*	2	25 May 1997	72^{II}
CV 166	*A. ceterach*	2	25 May 1997	4x
CV 167	*A. ceterach*	2	25 May 1997	4x
CV 168	*A. ceterach*	2	25 May 1997	
CV 169	*A. ceterach*	2	25 May 1997	4x
CV 170a	*A. ceterach*	2	25 May 1997	4x
CV 170b	*A. ceterach*	2	25 May 1997	72^{II}
CV 171	*A. octoploideum*	2	25 May 1997	8x
CV 172	*A. octoploideum*	2	25 May 1997	8x
CV 173	*A. octoploideum*	3	26 May 1997	8x
CV 175	*A. octoploideum*	3	26 May 1997	8x
CV 176	*A. octoploideum*	3	26 May 1997	8x
CV 177	*A. octoploideum*	3	26 May 1997	8x
CV 179	*A. octoploideum*	3	26 May 1997	8x
CV 188	*A. octoploideum*	3	28 May 1997	144^{II}
CV 180	*A. aureum*	4	26 May 1997	4x
CV 181	*A. aureum*	4	26 May 1997	4x
CV 182	*A. aureum*	4	26 May 1997	

APPENDIX 2. Vouchers from La Palma, with corresponding taxa, locality numbers, and chromosome numbers. CV is the abbreviation for Caroline Van den heede. The description of the localities and their number is given in Material and Methods. For samples with chromosomes counted, the number of bivalents is given. Counted specimens served as standards to determine the ploidy level (indicated by 4x and 8x) by flow cytometry. All specimens were used for isozyme analysis.

Voucher number	Taxon	Locality	Date of collection	Meiotic chromosome number (n), or ploidy
CV 708	A. octoploideum	5	2 Apr. 1999	8x
CV 709	A. octoploideum	5	2 Apr. 1999	144^{II}
CV 711	*A. aureum*	6	3 Apr. 1999	4x
CV 712	*A. aureum*	6	3 Apr. 1999	4x
CV 713	*A. aureum*	6	3 Apr. 1999	
CV 715	*A. octoploideum*	7	4 Apr. 1999	8x
CV 716	*A. octoploideum*	7	4 Apr. 1999	8x
CV 717	*A. octoploideum*	8	4 Apr. 1999	8x
CV. 718	*A. octoploideum*	8	4 Apr. 1999	8x
CV 719A	*A. octoploideum*	8	4 Apr. 1999	8x
CV 719B	*A. octoploideum*	8	4 Apr. 1999	8x
CV 720	*A. octoploideum*	8	4 Apr. 1999	8x
CV 721	*A. octoploideum*	8	4 Apr. 1999	8x
CV 723	*A. octoploideum*	9	4 Apr. 1999	8x
CV 724	*A. octoploideum*	9	4 Apr. 1999	8x
CV 725	*A. octoploideum*	10	4 Apr. 1999	8x
CV 726	*A. octoploideum*	10	4 Apr. 1999	8x
CV 727	*A. octoploideum*	10	4 Apr. 1999	8x
CV 729	*A. octoploideum*	11	5 Apr. 1999	8x
CV 730A	*A. octoploideum*	11	5 Apr. 1999	8x
CV 730B	*A. octoploideum*	11	5 Apr. 1999	8x
CV 731	*A. octoploideum*	12	5 Apr. 1999	144^{II}
CV 732	*A. octoploideum*	12	5 Apr. 1999	8x
CV 733	*A. octoploideum*	12	5 Apr. 1999	8x
CV 734	*A. octoploideum*	12	5 Apr. 1999	8x
CV 740	*A. octoploideum*	13	9 Apr. 1999	8x
CV 741	*A. octoploideum*	13	9 Apr. 1999	8x
CV 742	*A. octoploideum*	13	9 Apr. 1999	8x
CV 743	*A. octoploideum*	13	9 Apr. 1999	8x
CV 744	*A. octoploideum*	13	9 Apr. 1999	8x
CV 745	*A. octoploideum*	13	9 Apr. 1999	8x
CV 746	*A. octoploideum*	13	9 Apr. 1999	8x
CV 747	*A. octoploideum*	13	9 Apr. 1999	8x
CV 748	*A. octoploideum*	13	9 Apr. 1999	8x
CV 749	*A. octoploideum*	13	9 Apr. 1999	8x
CV 750	*A. octoploideum*	13	9 Apr. 1999	8x

APPENDIX 3. Vouchers from Tenerife, with corresponding taxa, locality numbers, and chromosome numbers. CV, RV, and WB are abbreviations for Caroline Van den heede, Ronald Viane, and Wilfried Bennert. The description of the localities and their number is given in Material and Methods. For samples with chromosome counted, the number of bivalents is given. Counted specimens served as standards to determine the ploidy level (indicated by 4x and 8x) by flow cytometry. All specimens were used for isozyme analysis.

Voucher number	Taxon	Locality	Date of collection	n or ploidy
RV 6135	*A. ceterach*	14	7 May 1995	72^{II}
CV 183	*A. ceterach*	14	27 May 1997	72^{II}
CV 184	*A. ceterach*	14	27 May 1997	72^{II}
CV 185	*A. ceterach*	14	27 May 1997	4x
CV 186	*A. ceterach*	14	27 May 1997	72^{II}
CV 187a	*A. ceterach*	14	27 May 1997	72^{II}
CV 187b	*A. ceterach*	14	27 May 1997	4x
CV 187c	*A. ceterach*	14	27 May 1997	4x
CV 663B	*A. ceterach*	14	11 Jan. 1999	4x
CV 665	*A. aureum*	15	12 Jan. 1999	4x
CV 666	*A. aureum*	15	12 Jan. 1999	4x
CV 667	*A. aureum*	16	12 Jan. 1999	
CV 668	*A. aureum*	16	12 Jan. 1999	
CV 669	*A. aureum*	16	12 Jan. 1999	4x
CV 670	*A. aureum*	16	12 Jan. 1999	72^{II}
CV 671	*A. aureum*	16	12 Jan. 1999	4x
CV 672A	*A. octoploideum*	17	13 Jan. 1999	8x
CV 672B	*A. octoploideum*	17	13 Jan. 1999	8x
CV 674	*A. octoploideum*	17	13 Jan. 1999	8x
CV 675	*A. aureum*	18	14 Jan. 1999	4x
CV 676	*A. aureum*	18	14 Jan. 1999	4x
CV 677	*A. aureum*	18	14 Jan. 1999	4x
CV 678	*A. aureum*	18	14 Jan. 1999	4x
CV 683	*A. ceterach*	19	15 Jan. 1999	4x
CV 684	*A. ceterach*	19	15 Jan. 1999	4x
CV 686	*A. octoploideum*	19	15 Jan. 1999	8x
CV 687	*A. octoploideum*	19	15 Jan. 1999	8x
CV 695	*A. octoploideum*	19	15 Jan. 1999	8x
CV 696	*A. ceterach*	19	15 Jan. 1999	4x
CV 701	*A. ceterach*	20	15 Jan. 1999	4x
CV 702A	*A. ceterach*	20	15 Jan. 1999	4x
CV 702B	*A. ceterach*	20	15 Jan. 1999	4x
CV 704	*A. ceterach*	20	15 Jan. 1999	4x
CV 705	*A. aureum*	21	16 Jan. 1999	4x
CV 706	*A. aureum*	21	16 Jan. 1999	4x
CV 707	*A. aureum*	21	16 Jan. 1999	4x
CV 751A	*A. octoploideum*	22	10 Apr. 1999	8x
CV 751B	*A. octoploideum*	22	10 Apr. 1999	
CV 752	*A. octoploideum*	22	10 Apr. 1999	8x
CV 754A	*A. octoploideum*	23	10 Apr. 1999	8x
CV 754B	*A. octoploideum*	23	10 Apr. 1999	8x
CV 754C	*A. octoploideum*	23	10 Apr. 1999	8x
WB 22/93	*A. octoploideum*	24	15 Apr. 1993	144^{II}

APPENDIX 4. Alphabetical list of material for comparison used in this study. CV, RV, TR, and WB are abbreviations of C. Van den heede, R. Viane, T. Reichstein, and W. Bennert. Vouchers are deposited in GENT and in our personal herbarium at Ghent University. Additional information about localities is available from the first and the last author (lienvdheede@hotmail.com; ronnie.viane@UGent.be). Voucher information about 108 Cypriot samples is given in Van den heede et al. (2002).

Asplenium ceterach:	
CV25b+c	Slovenia, Kal
CV25b, CV30a+b, CV31	Croatia, Roč
CV36a+b, CV37	Croatia, Bassania
CV38b	Slovenia, Korte
CV41,CV42b, CV44,CV48, CV49b	Italy, Valle della Marossa
CV64, CV65, CV66, CV67, CV494	Italy, Termine di Roverano
CV225	Cyprus, Troodos Mts., Chandria
CV275a+b, CV276, CV277	Belgium, Marcourt
CV278, CV279	Italy, Cannero Riviera
CV281a+b, CV282, CV283, CV285, CV286	United Kingdom, Wales, Snowdonia
CV429, CV430, CV431	Crotia, Losinj
CV445, CV448, CV449	Italy, Berceto
CV450, CV451	Italy, Boio
CV657, CV658, CV659	Spain, Torrelodones
CV767, CV768, CV769, CV770, CV771	Spain, Alava
CV773	France, Coulgens
CV774, CV775, CV776	France, Paulmy
RV5900	France, NNE of Montpellier, La Pene
WB1b+e/97	Turkey, Karaoba
WB10d/97	Turkey, Manisa
WB5c/97	Turkey, Okçular
WB12c+d/97	Turkey, Mugla
Asplenium cyprium:	
CV213	Cyprus, Troodos Mts., Tsakistra-Vroiska road
CV249	Cyprus, Kyrenia Mts., Kyrenia-Kythrea road
Asplenium dalhousiae	
CV318	Ethiopia, Harerge Province, Asbe Teferi
TR7634	Pakistan, Swat Province, Ambela
Asplenium javorkeanum:	
CV3, CV4, CV5	Italy, Stupizza
CV7a+b+c, CV8a+b+c	Slovenia, 1 km E of Bača towards Podbrdo
CV10, CV11, CV12, CV14, CV412, CV414	Slovenia, Bača-valley, Kneža-Klavže road
CV20a+b+c+d, CV21a+b+c+d+e	Slovenia, Matavun
CV81, CV82a+b, CV83, CV84, CV85a+b	Italy, Arni
CV86, CV87, CV88	Italy, Monte Freddone, 730m
CV89, CV90, CV91, CV92, CV93	Italy, Monte Freddone, 1320m
CV94a+b, CV95	Italy, Monte Freddone, 970m
CV404, CV405	Slovenia, Bovec-Kobarid road
CV410	Slovenia, Ljubinj
CV480	Italy, N slope of Pania Secca
CV483, CV484	Italy, Fosso di Antona
CV504, CV506	Italy, E slope of Monte Corchia
Asplenium lolegnamense:	
CV985	Madeira, SW slope of Pico Ruivo
CV993	Madeira, N of Serra de Agua

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Shorter Notes

Nomenclatural Corrections in *Adiantum*.—Two Brazilian species of *Adiantum* recently published by Prado in two different papers and journals need corrections. It has been brought to my attention that *Adiantum pulcherrimum* Prado is a later homonym of *A. pulcherrimum* Copel. (Philipp. J. Sci. C. 6:138, tab. 22. 1911), a fern that occurs in Borneo. Consequently, a new name is required for the Brazilian species:

Adiantum mynssenae Prado, *nom. nov.* Replaced synonym: *Adiantum pulcherrimum* Prado, Amer. Fern J. 93:76, fig. 1. 2003, *non* Copel. (1911).

The species is named for Claudine Mynssen, currently doing research on pteridophytes at the Jardim Botânico do Rio de Janeiro (RB Herbarium) and collector of the holotype. During my last visit to RB she called my attention to this beautiful species from the Atlantic forest. It is in recognition of her friendship and invaluable assistance that I name it for her.

Adiantum giganteum was published by Prado (Fern Gazette 16:209, figs. 1, 2. 2001), based on a holotype collected by Ynes Mexia from the state of Pará, Brazil, and cited as *Mexia 6031* (UC). However, the correct number of the holotype is *Mexia 6013* (UC). I thank Alan R. Smith and Layne Huiet, both UC, for directing my attention to these problems and for Smith's suggestions on an initial draft of this note.—Jefferson Prado, Instituto de Botânica, C. P. 4005, 01061-970, São Paulo – SP, Brazil.

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Distribution, Ecology and Cytology of *Asplenium azoricum* Lovis, Rasbach & Reichstein (Aspleniaceae, Pteridophyta) and Its Hybrids

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ABSTRACT.—New data on distribution, ecology and cytology of the Azorean endemic *Asplenium azoricum* are presented. Hybrids involving this species are recorded for the first time. The extremely rare, natural hybrid *A. azoricum* × *A. scolopendrium* is described as *A.* ×*santamariae*. It has been found at a single location on Santa Maria Island and is presumed to be triploid. A more frequent natural hybrid *A. azoricum* × *A. onopteris*, found at seven locations on the same island, is also new to science and is described here as *A.* ×*diasii*. It is confirmed cytologically as triploid with two genomes from *A. azoricum* and one from *A. onopteris*. *Asplenium* ×*diasii* and an experimental hybrid show that *A. azoricum* is an allotetraploid species. The parentage and directionality of hybridization for both hybrid taxa have been established using uniparentally inherited plastid genome markers.

The Azores is an isolated archipelago of nine inhabited, volcanic islands in the Northern Atlantic Ocean. The shortest distance to the European coast, Cabo da Roca, Portugal, is almost 1,300 km. The American coast, Newfoundland, is about 1,700 km distant (Fig. 1).

Four morphologically similar, simply-pinnate species of the genus *Asplenium* have been reported in the Azores: the almost cosmopolitan tetraploid *A. trichomanes* L. ssp. *quadrivalens* (D. E. Mey.) Lovis; the widely scattered Neotropical triploid apomict *A. monanthes* L.; the locally rare Macaronesian endemic diploid *A. anceps* Lowe ex Hook. & Grev. (Rasbach *et al.*, 1981), two plants of which were reported from Pico island in 1973 (Lovis *et al.*, 1977) and the endemic tetraploid *A. azoricum* Lovis, Rasbach & Reichstein.

Although *A. azoricum* was collected by K. Hochstetter in 1838 on Faial Island (Seubert, 1844), its specific distinction was not recognized until the work of Lovis *et al.* (1977). Key distinguishing features had already been commented upon by Wilmanns and Rasbach (1973), and earlier authors, e.g., Milde (1867), and Trelease (1897), obviously recognized that Azorean material differed from typical *A. anceps*, referring their gatherings to a forma (f. *azorica* Milde) of that species. While previously confused with both *A. anceps* and *A. trichomanes*, its separation in the field generally is quite easy. *Asplenium azoricum* differs from both in the more elongated, broadly-triangular, often

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biauriculate, more conspicuously dentate pinnae, and from the latter in its glossier, bright green frond colour. Typical luxuriant material from shaded, sheltered environments is considerably larger than any of the taxa listed above, with fronds sometimes exceeding 35 cm long. Confusion is possible, particularly with *A. trichomanes* ssp. *quadrivalens*, when comparing small examples from dry, exposed environments. *Asplenium anceps*, which is implicated in the parentage of *A. azoricum* but currently is not sympatric with it, differs in having a very pronounced additional third rachis wing on the abaxial side. A greater potential confusion is with another recently described taxon, *A. trichomanes* ssp. *coriaceifolium* H. & K. Rasbach, Reichstein & Bennert (syn.: *A. azomanes* Rossello, Cubas & Rebassa). This highly restricted tetraploid was described from walls terracing olive groves in the Balearic Islands, where it is also found in sheltered rocky gulleys of seasonal watercourses close to sea-level. It, however, also occurs on montane karstic limestones in Southern Spain and the Rif mountains of Morocco (Rumsey and Vogel, unpubl.). Its genomic constitution and relationship to *A. azoricum* are currently being explored.

Natural hybrids of *A. azoricum* have not previously been reported (Reichstein, 1981) although they have been actively searched for (Lovis *et al.*, 1977). Plastids have been shown to be maternally inherited in *Asplenium* (Vogel *et al.*, 1998a), and fragment length polymorphism and sequence data for this moderately variable plastidic region allows for unequivocal identification to the species level (Vogel *et al.*, unpubl.). Thus, molecular studies of this sort facilitate both the determination of hybrid parentage and the establishment of hybrid directionality, i.e., which parental taxon was maternal.

Material and Methods

The distribution and ecology of *A. azoricum* was studied between 1998 and 2001 on the nine islands of the archipelago, during field work by H.S. for a project mapping all vascular plant species. Distribution maps based on the UTM 1 × 1 km grid have been created for islands representative of the western, central and eastern groups of the archipelago: Flores, Faial and Santa Maria (Fig. 2).

During the course of our fieldwork, a number of plants with abortive spores were detected whose morphology suggested possible hybridity. Fronds with premature sporangia were fixed in a mixture of acetic acid and ethanol (1:3) in the field. Preparations of meiosis were made following Manton (1950). A few mature fronds of each potential hybrid were collected as voucher specimens and will be deposited in the herbaria of Universidade dos Açores, Terceira (AZU) and Natural History Museum, London (BM). Live material was not collected as all hybrids are rare and should be protected in their natural habitat on the islands.

Total DNA extractions were made from small (c. 20 mg) portions of each of the herbarium specimens using the method of Rogers & Bendich (1994) and amplified using the universal plastid primers C and F of Taberlet *et al.* (1991).

FIG. 1. Location of the Azores in the Atlantic Ocean.

RESULTS

ASPLENIUM AZORICUM LOVIS, RASBACH & REICHSTEIN.—*Asplenium azoricum* (Fig. 5a) was found on all nine islands of the archipelago, the only member of the *A. trichomanes* group for which this is true. Unlike *A. trichomanes* ssp. *quadrivalens*, which in the Azores is largely restricted to human-made structures and most abundant on the most populous islands, *A. azoricum* is widely distributed in a range of natural and semi-natural environments. Although it has been able, like many rock ferns, to exploit built structures it is not restricted to them. Usually a species of somewhat humid environments, paradoxically it is most common and shows its widest ecological amplitude on the driest, easternmost island of Santa Maria. Here it effectively replaces *A. trichomanes* ssp. *quadrivalens* (a species with very few individual plants on the island), existing in large numbers from coastal areas up to c 575 m a.s.l. on Pico Alto (Fig. 2).

Throughout the archipelago, the species achieves its greatest abundance at lower altitudes, declining markedly above the lowland forest zone. Elsewhere in the eastern group, it is locally frequent and often luxuriant in the central low-lying valley between the major volcanic peaks of São Miguel Island. In the central group of the Azores it is more restricted to west and north-west exposed

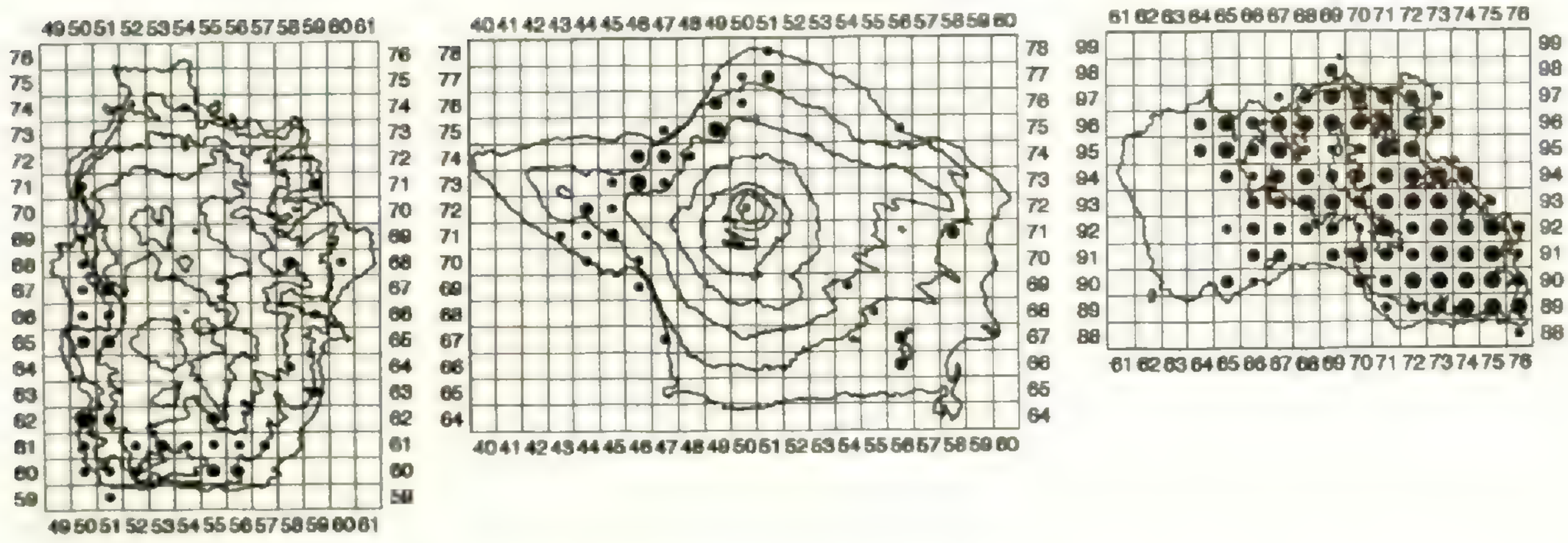

FIG. 2. Distribution of *A. azoricum* on the islands of Flores (left), Faial (center), and Santa Maria (right) in UTM 1 × 1 km grid, WGS 84 (contour lines along 200 m isohypses). Symbol size indicates abundance within that 1 × 1 km square: rare •, occasional • or common ●.

slopes between 100–500 m a.s.l., although it ascends to at least 700 m on Pico (*Rumsey et al. 96-10-3-3*, BM!). In the western group, Flores and Corvo Islands, *A. azoricum* exists in the absence of *A. trichomanes* ssp. *quadrivalens*. On Flores, it is widespread (see Fig. 2) but uncommon; on Corvo, it should be considered very rare. On these islands, the species is usually found in small populations (<20 individuals), or as single plants and only at altitudes below 500 m. Restriction to particular site aspects is not so marked as in the central group, presumably because the more generally humid climate reduces the need for shelter.

Asplenium azoricum is most common on steep, humid slopes of shady ravines at low altitudes. In these places it forms large populations on bare soil, often mixed with other ferns like *A. scolopendrium* or *A. onopteris*. However, judging by earlier literature reports (e.g. Lovis *et al.*, 1977) and herbarium gatherings, it is most likely to be found on or at the base of walls by roadsides and field-margins. It is one of several shade tolerant fern species that predominantly occurs in lowland areas dramatically influenced by the almost complete destruction of the natural vegetation following human arrival on the islands in the 15th and 16th centuries. *Asplenium azoricum* has faired better than some, and is now one of the very few native species that can be found in lowland forests and plantations dominated by the Australian neophyte *Pittosporum undulatum*. In this environment, it occurs in deep shade in small soil pockets on volcanic rocks on the forest floor, or rarely on the ground. In the coastal zone, it is absent in sea-wind exposed sites but grows in stands of *Erica azorica* or *Arundo donax* together with *Asplenium marinum*. Inland, while often found on mossy rocks at the base of walls, it can also be found higher on the wall proper, although typically only in soil filled crevices. A requirement for humidity throughout the year means it usually is absent from dry, south-exposed or mortared walls where it is replaced by the more xerophytic and calcicolous *A. trichomanes* ssp. *quadrivalens*. Soil backed retaining walls, or old walls which have accumulated substrate in the

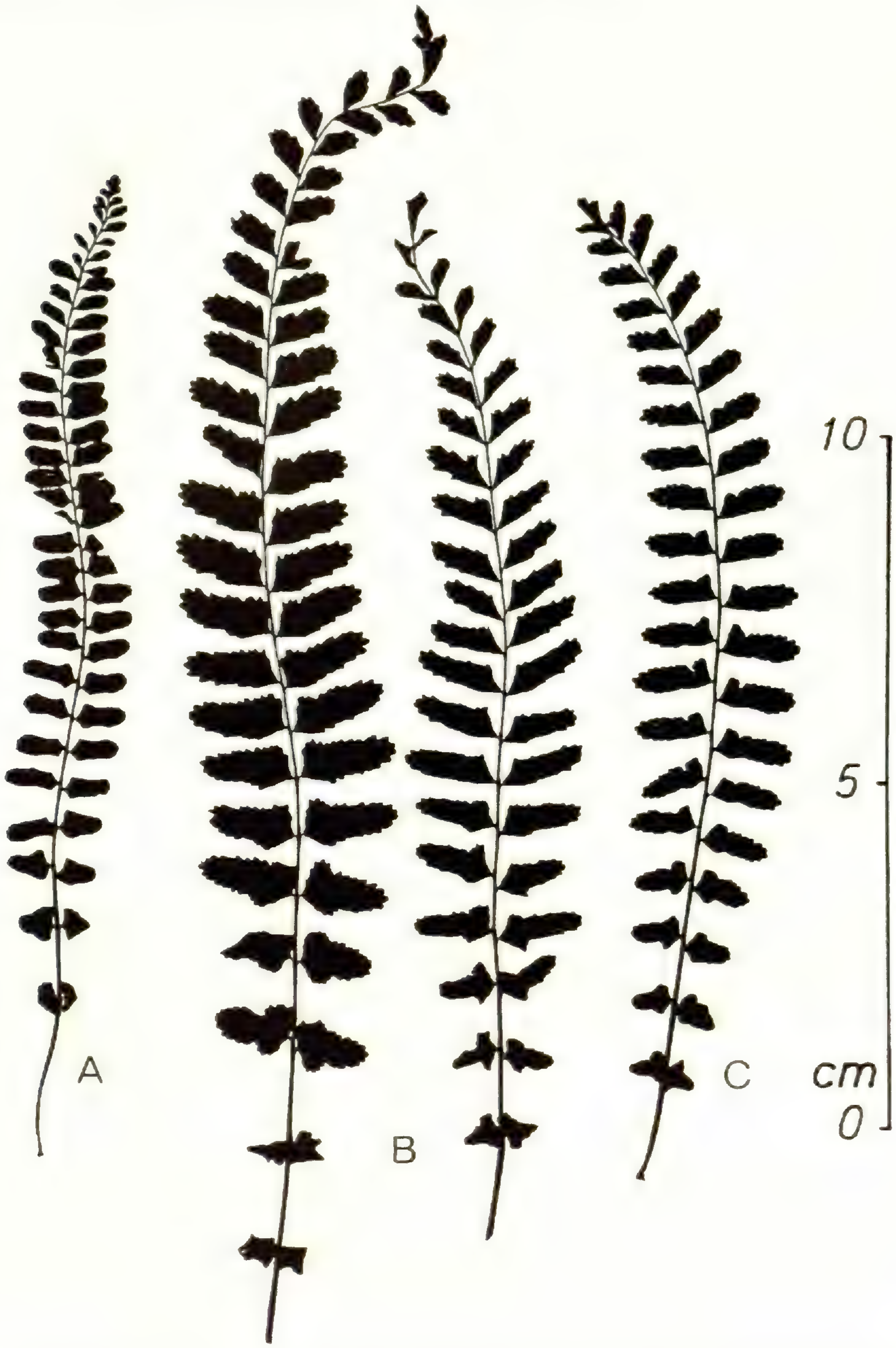

FIG. 3. Silhouettes of fronds. A. *Asplenium anceps* (Madeira, TR-2559). B. Experimental hybrid *A. anceps* × *A. azoricum* (*TR-5188* and *5188/7*, in B 200125235/37, col. *H. & K. Rasbach*). C. *A. azoricum* (Azores, *TR-3335/2*). A and C from *Lovis et al.*, 1977; B- photo H.R.; TR stands for T. Reichstein.

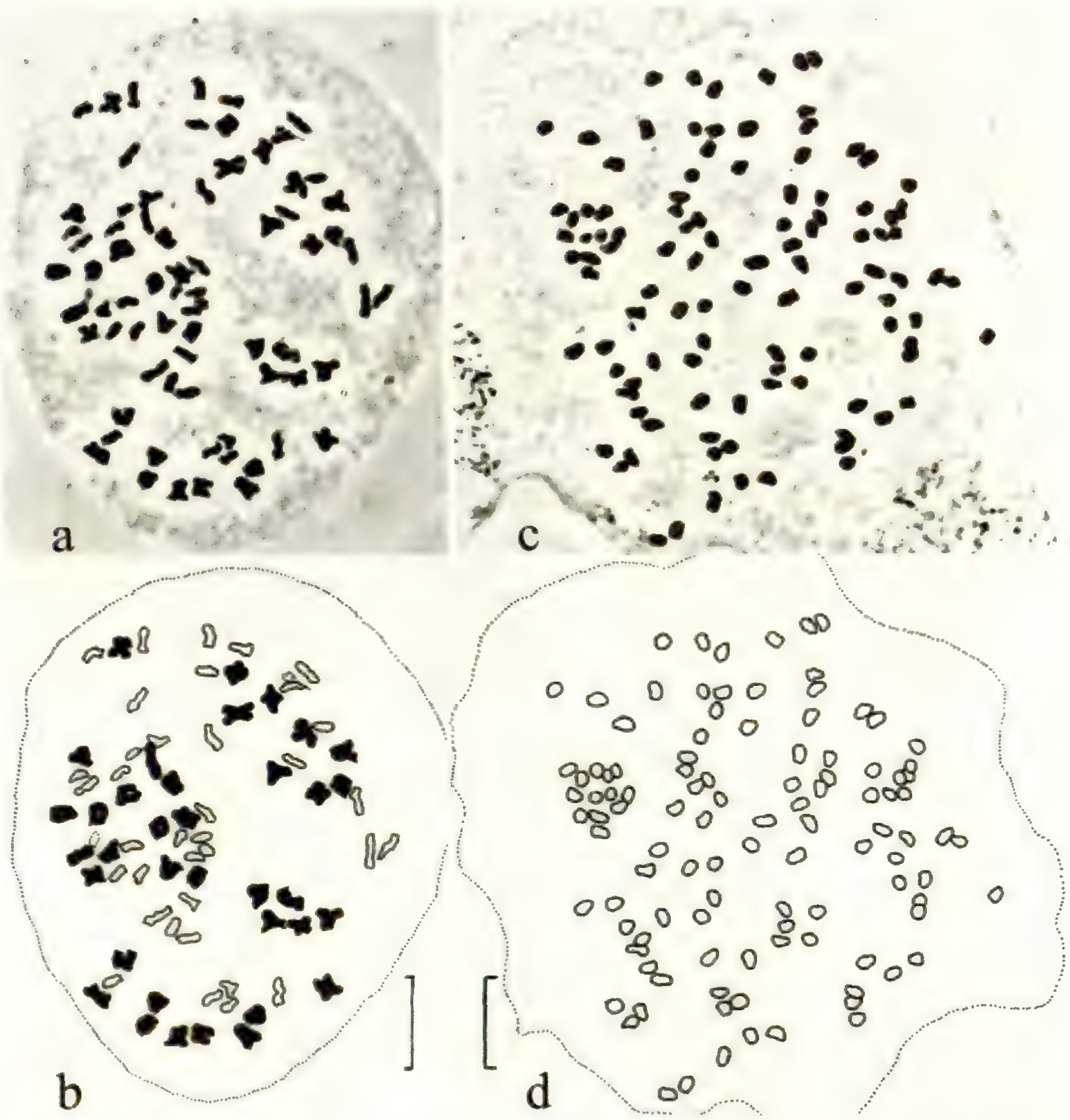

FIG. 4. A. Cytology of *Asplenium*. a. Photomicrograph of *A. anceps* × *A. azoricum*; spore mother cell in meiosis with n = 36II and 36I. b. Explanatory diagram of a; scale bar 10 μm, pairs black, univalents outlined (*TR-5188*, in B, collection *H. & K. Rasbach*: 200125235/37). c. Photomicrograph of *A.* ×*diasii*; spore mother cell in meiosis with n = 108I; d. Explanatory diagram of c; scale bar 10 μm; prep. and photographs (HR *Az-Ma-927 Schäfer*).

interstices and are partially shaded by other vegetation, are thus most likely to support the endemic taxon. In summary, the ecological range of *A. azoricum* seems to be rather broad and with the few existing relevés it is not possible to describe vegetation associations.

Cytological investigations by Lovis *et al.* (1977) showed that *A. azoricum* is tetraploid with 2n = 144. To determine the nature of its polyploidy and relationship to other Macaronesian taxa, a program of experimental hybridization

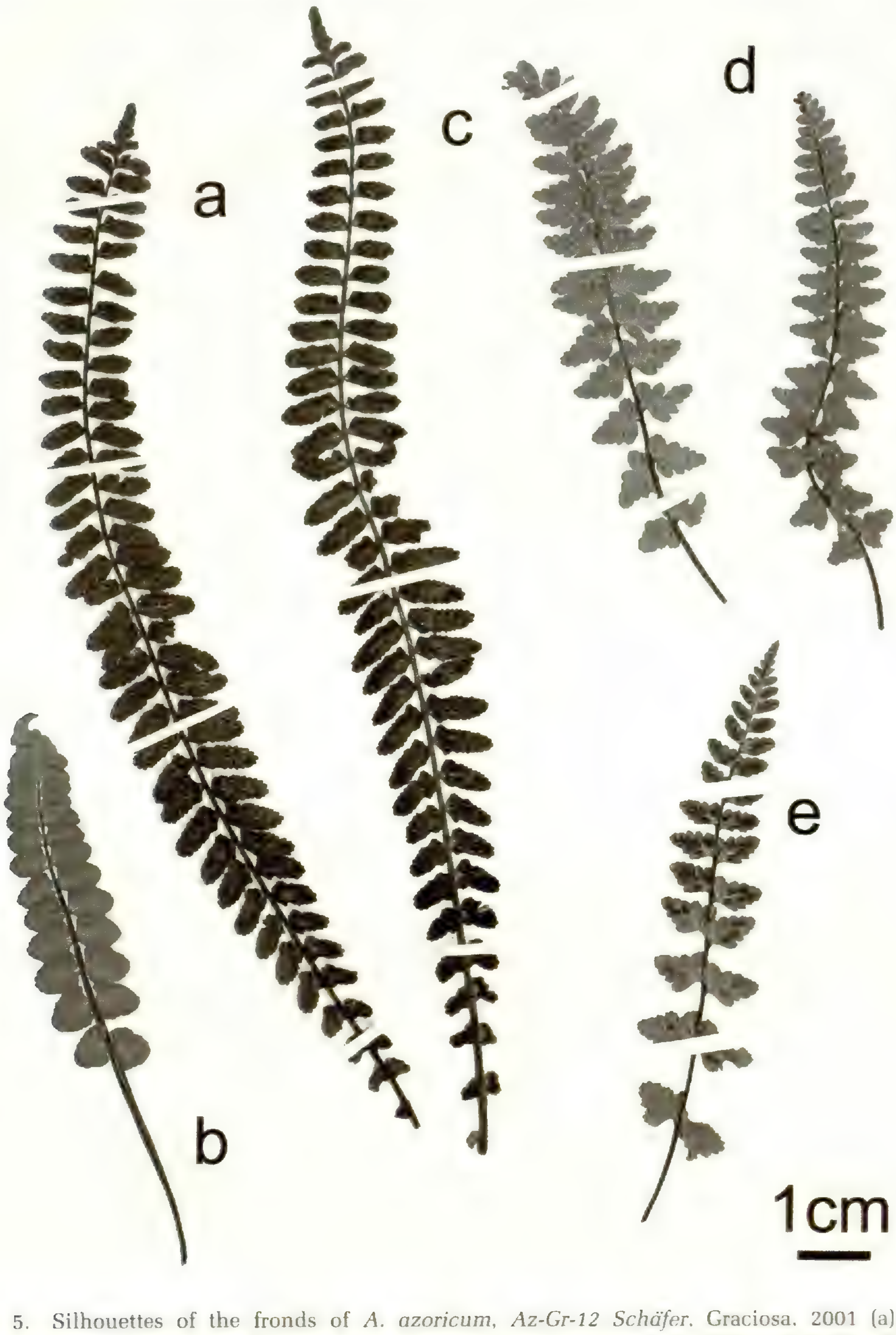

FIG. 5. Silhouettes of the fronds of *A. azoricum*, *Az-Gr-12 Schäfer*, Graciosa, 2001 (a), *A. ×santamariae*, *Az-Ma-1064 Schäfer*, Santa Maria, 2001 (b), and *A. ×diasii*, *Az-Ma-749 Schäfer*, Santa Maria, 2001 (c), *Az-Ma-915 Schäfer*, Santa Maria, 2001 (d), *Az-Ma-1036 Schäfer*, Santa Maria, 2001 (e).

and cytological investigation was begun. In 1978, the late Prof. T. Reichstein hybridized *A. anceps* Lowe *ex* Hooker & Grev. from Madeira and *A. azoricum* from the Azores (for method see Rasbach *et al.*, 1994). This hybridization program produced 35 plants of *A. anceps*, five plants of *A. azoricum*, and 22 hybrids (fig. 3). Cytological study of one of these hybrids by Prof. J. J. Schneller (Zürich) revealed that it was triploid with n = ca. 36 II and 36 I (Schneller, in litt. 23.01.1995). As part of this study H. Rasbach analyzed meiosis in another hybrid plant. The chromosome number of ten spore mother cells was n = ca. 36 II and 36 I (fig. 4A).

The most parsimonious explanation of this pairing behavior is that tetraploid *A. azoricum* shares a genome with *A. anceps* and that these homologous n chromosome sets undergo synapsis in the hybrid to form the 36 pairs. The 36 unpaired chromosomes would thus represent a non-homologous genome contributed by the second diploid parent of *A. azoricum*. Under this hypothesis, *Asplenium azoricum* is an allotetraploid and chromosome pairing observed in the hybrid *A. anceps* × *A. azoricum* is allosyndetic. The alternative hypothesis, that *A. azoricum* is an autopolyploid and that pairing in the hybrid is autosyndetic (occurring between the two genomes contributed by *A. azoricum*) can be rejected based on cytological study of the natural hybrid *A.* ×*diasii* reported below. We therefore conclude that *A. anceps* is one of the parental species of *A. azoricum* and suggest that the genomic constitution of this allotetraploid be represented by the formula AnAnUnUn, where An = *anceps* and Un is unknown.

On morphological grounds we hypothesize that the unknown parent of *A. azoricum* is a member of the *A. trichomanes* group *sensu lato*. Allozyme and DNA studies (Vogel *et al.*, unpublished) preclude the possibility that the missing parent (UnUn) is an extant European taxon. Lovis *et al.* (1977) commented on the morphological similarities with the Neotropical *A. heterochroum* Kunze. However, the material of this or similar species they examined was tetraploid and hexaploid (unpublished records), not diploid as would be required of the missing parent. Although the missing parent remains unknown, the likelihood of a Neotropical origin is perhaps strengthened by the growing list of Macaronesian cryptogamic species disjunct to the Neotropics, or with their closest relatives there. These include the pteridophytes *Ceradenia jungermannioides* (Klotzsch) R. C. Ching, *Grammitis marginella* (Sw.) Sw. (Schäfer, 2001), *Isoëtes azorica* Dur. *ex* Milde (Britton and Brunton, 1996), and *Huperzia dentata* (Herter) J. Holub, and the bryophytes *Jamesoniella rubricaulis*, *Radula nudicaulis* (Sjögren, 2000), *Plagiochila retrorsa* Gottsche, *P. virginica* A. Evans, *P. stricta* Lindenb., *P. papillifolia* Steph. and *P. longispina* Lindenb. & Gottsche (Rycroft, 2002).

Asplenium* ×*diasii Schaefer, Rumsey & Rasbach, *hybr. nov.* (*Asplenium azoricum* Lovis, Rasbach & Reichstein × *A. onopteris* L.) TYPE:—Açores (Portugal), Ilha de Santa Maria, São Lourenço, 150 m a.s.l., in *Pittosporum undulatum* forest, n = 108I, triploid. 01.08.2001, *H. Schäfer, Az-Ma-749* (holotype, BM). **Fig. 4c–d, 5c–e.**

Planta hybrida Asplenio azorico simillima; sed rhizoma caespitosa; petiolus rachisque basi ferrugineo-fuscis apice viridibus; folia 6–18 cm longa, 3–3.5 cm lata, lanceolato-acuminata, basi bipinnata; pinnae oblongo-ovatae, brevissime petiolatae, margine dentatae; sporae omnes abortivae; chromosomatum numerus 108, meiosi chromosomatibus 108 univalentibus; differt.

Rhizome caespitose. Fronds 6–18 cm, stipe to 5 cm, stipe and rachis black or dark reddish brown, the uppermost 2 cm green. Pinnae numerous (ca. 15–37), up to 16 × 12 mm, light green, short petiolate, entire or the lower bipartite, triangular to oblong-ovate, margin sharply dentate. Sori ca 1.0–3.3 mm. Triploid hybrid of the tetraploid *A. azoricum* and the diploid *A. onopteris* (maternal parent). The hybrid contains one genome of *A. onopteris* and two of *A. azoricum* that do not form pairs, i.e. are not homologous.

Named after the Azorean botanist Prof. Dr. Eduardo Dias.

PARATYPES.—Açores (Portugal), Ilha de Santa Maria: Feteiras de Cima, 220 m a.s.l., roadside slope in pasture, 13.06.2001, *H. Schäfer Az-Ma-915* (AZU, BM) Fig 5d; Loural, 350 m a.s.l., *Erica* shrub on roadside, 15.06.2001, *H. Schäfer Az-Ma-926* (AZU, BM); Loural, 350 m a.s.l., W exposed slope in pasture, n = 108I, 15.06.2001, *H. Schäfer Az-Ma-927* (AZU, BM), (Fig. 4c); Açores (Portugal), Ilha de Santa Maria, Loural, 350 m a.s.l., *Erica* shrub on roadside, n = 108I,15.06.2001, *H. Schäfer Az-Ma-928* (AZU, BM); Loural, 360 m a.s.l., *Erica* shrub on roadside, 15.06.2001, *H. Schäfer Az-Ma-929* (AZU, BM); Loural, 360 m a.s.l., *Erica* shrub on roadside, 15.06.2001, *H. Schäfer Az-Ma-1200* (AZU, BM); NE Cruz dos Picos, roadside slope in pasture, 22.06.2001, *H. Schäfer Az-Ma-983* (AZU, BM); Santa Bárbara, 200 m a.s.l., *Pittosporum undulatum* forest on roadside slope, 17.07.2001, *H. Schäfer Az-Ma-1036* (AZU, BM) Fig. 5e; Feteirinha, 310 m a.s.l., E exposed slope in pasture, 24.07.2001, *H. Schäfer Az-Ma-1072* (AZU, BM); Cardal, 350 m a.s.l., SW exposed slope in pasture, 05.08.2001, *H. Schäfer Az-Ma-1108* (AZU, BM).

As the only simply pinnate member of the *A. trichomanes* group present in the vicinity of these hybrids, *A. azoricum* is almost certainly one of the parents. The other parent was less easily determined. Because *Asplenium* hybrids generally show clear morphological intermediacy (Wagner, 1954), the second parent was presumed to have more dissected, bi- to tripinnate fronds. In the field it was assumed that at least some of the hybrid plants could have *A. obovatum* Viv. ssp. *lanceolatum* (Fiori) P. Silva as the second parent, it being present in the majority of hybrid sites. Subsequent cytological examination revealed that the three plants investigated were triploid, not tetraploid, as would be expected from a hybrid between tetraploid *A. azoricum* and tetraploid *A. obovatum* ssp. *lanceolatum*.

Further investigation was therefore needed to establish the identity of the second parent(s). DNA extraction and PCR amplification of a portion of the plastid genome was carried out on five of the individuals identified as hybrids: *HS Az-Ma 749, 927, 928, 1014* and *1036*. Each generated identical fragments of c 1000 bp which differed markedly in their base sequence from both *A. obovatum* and *A. azoricum* but which matched that of *A. onopteris*, another

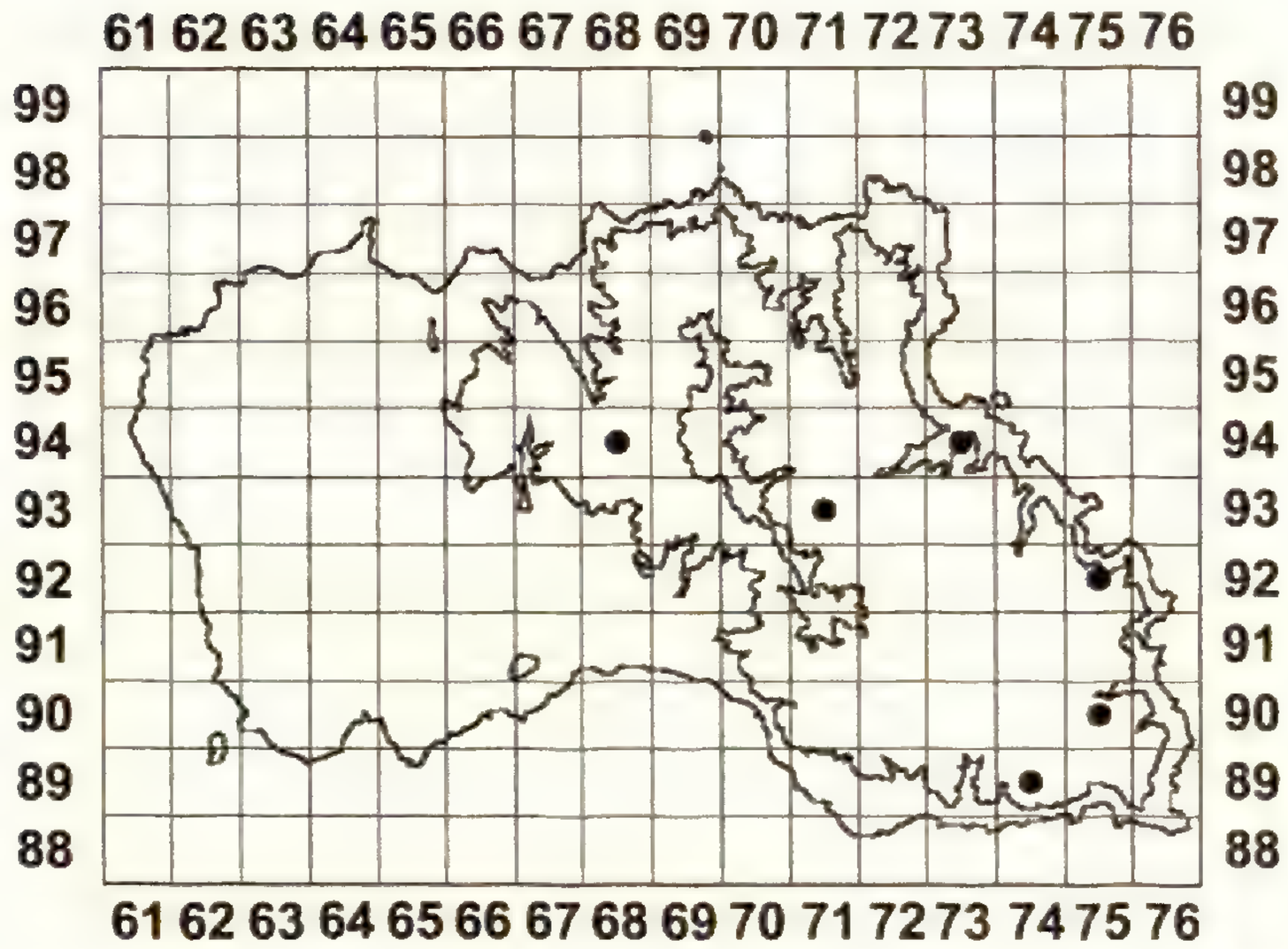

FIG. 6. Distribution of *Asplenium* ×*diasii* on Santa Maria island in UTM 1 × 1 km grid, WGS 84 (contour lines along 200 m isohypses).

species present at the majority of hybrid sites. The second (and consistently maternal) parent of *A.* ×*diasii* is therefore concluded to be *A. onopteris*. This fits with the cytological data because *A. onopteris* is diploid. The considerable variability of *A. onopteris* may help explain the range of morphologies shown by the hybrid, *A. azoricum* being rather invariable. Strong unidirectionality in hybrid formation has previously been reported for *A.* ×*alternifolium* (Vogel *et al.*, 1998b) and is true for a range of hybrids in which *A. onopteris* and its polyploid derivatives take part (Rumsey and Russell, unpubl.).

Altogether, 13 individual plants of this hybrid were found in seven locations of the eastern part of Santa Maria Island (Fig. 6). It has yet to be detected on any other island of the archipelago. The likelihood of the formation of this hybrid on the other islands is undoubtedly lessened by the reduced abundance of *A. azoricum* elsewhere. We might speculate that as the paternal species, hybrid formation is dependent on the presence of extensive growths of *A. azoricum* gametophytes and limited numbers of those of the maternal parent such that the ratio of *A. azoricum* to other species' antherozoids favors interspecific matings.

The hybrids were usually found in large mixed populations of the parents. Hybrid plants were restricted to more humid situations than the parents, often growing below them and near the foot of slopes, in communities dominated by *Pittosporum undulatum*, or *Erica azorica*.

Asplenium ×santamariae Schaefer, Rumsey & Rasbach, *hybr.nov.* (*Asplenium azoricum* Lovis, Rasbach & Reichstein × *A. scolopendrium* L.). TYPE:—Açores (Portugal), Ilha de Santa Maria, Santo Espírito, 280 m a.s.l., W. exposed slope in pasture. 21.07.2001, H. Schäfer, Az-Ma-1064, (holotype, BM; isotype, AZU). **Fig. 5b**

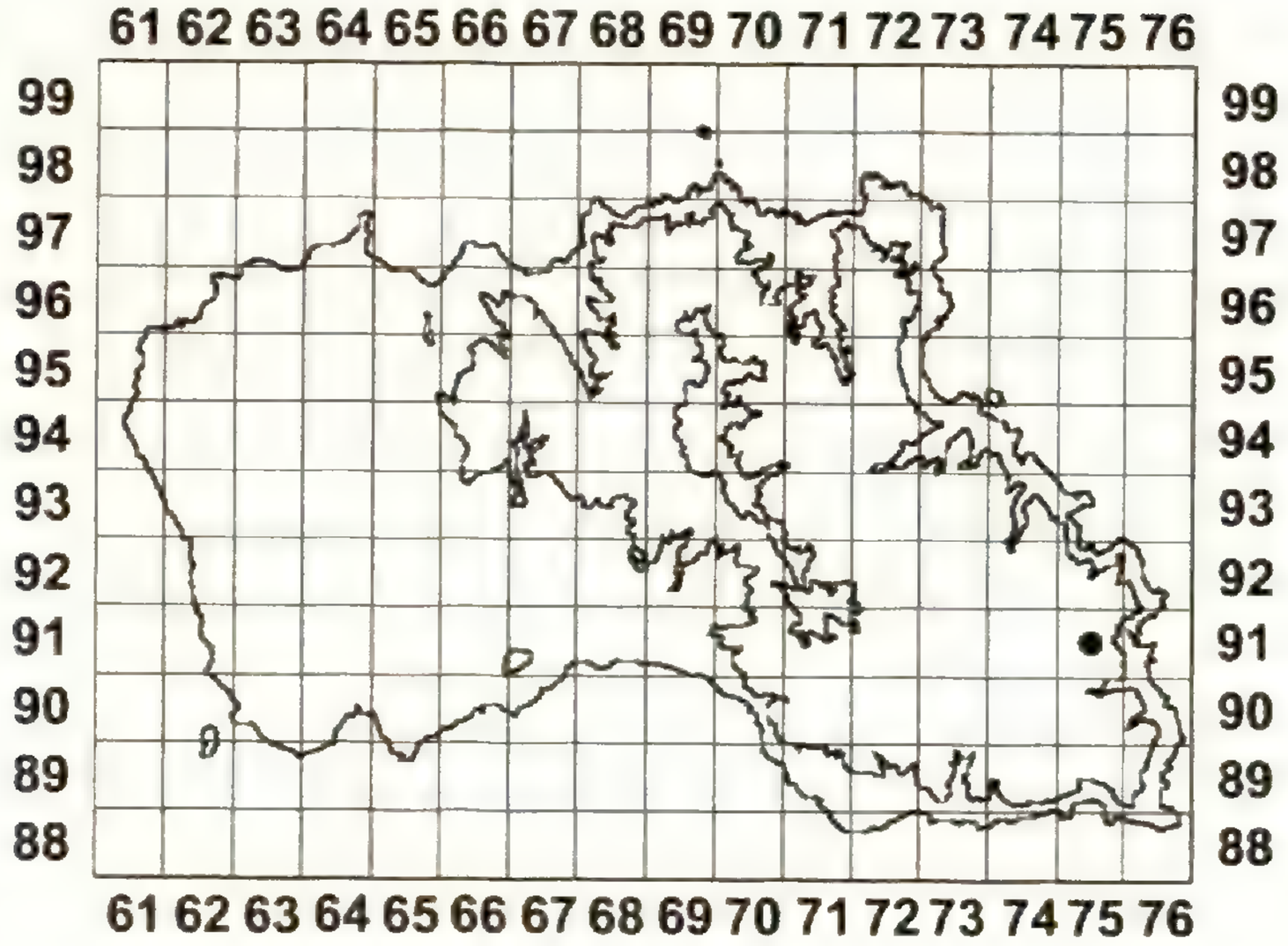

FIG. 7. Distribution of *Asplenium* ×*santamariae* on Santa Maria island in UTM 1 × 1 km grid, WGS 84 (contour lines along 200 m isohypses).

Planta hybrida ex *A. azoricum* et *A. scolopendrium* exorta; rhizoma caespitosa; folia 10–15 cm longa, 2 cm lata; petiolus 3–5 cm longus, fuscus; rachis basi ferrugineo-fusca; pinnae cordatae-ovatae, 10 × 9 mm, crispatae; superiores approximatae; sporae omnes abortivae.

Fronds 10–15 cm; stipe ca 5 cm, rachis ca 8 cm, black except uppermost 2 cm; pinnae up to 33, slightly crispate, confluent and crowded towards the tip of the frond; margin entire to shallowly dentate up to 10 × 9 mm, ovate-elliptical to cordate, light green; sori ca 1 mm. Hybrid of *A. azoricum* and *A. scolopendrium* (the latter the maternal parent) with abortive spores. Given the ploidy levels of the two parents this taxon is assumed to be triploid. Fig. 5b.

In appearance *A.* ×*santamariae* very closely resembles *A.* ×*confluens* T. Moore, the extremely rare hybrid between *A. trichomanes* ssp. *quadrivalens* and *A. scolopendrium*. It differs from that hybrid in its less distinctly stalked pinnules with more crenulate-dentate margins and a somewhat thicker overall texture. As with *A.* ×*diasii*, it is clear that the *A. trichomanes* group parent present in this hybrid is *A. azoricum*, it being the only simply pinnate species present. The confluent tip and broader, more decurrent pinnule attachments clearly indicate that the second parent of this hybrid would have fronds less divided than that of *A. azoricum*. The only Macaronesian species with less divided fronds are *A. scolopendrium* and *A. hemionitis* L. The latter is very rare on Santa Maria and has never been reported to hybridize with other species. It has a very distinctive, acutely lobed, palmate leaf, no sign of which is present in *A.* ×*santamariae*. The participation of *A. scolopendrium*, in the origin of *A.* ×*santamariae* is confirmed by examination of the hybrid's plastid genome.

Only one small individual of this hybrid was found in the archipelago. It grows in the south-eastern part of Santa Maria island (Fig. 7). The hybrid was

found in a large mixed population of the parents growing on a steep, west exposed slope with some *Pittosporum undulatum*. The slope and the *Pittosporum* stands are part of a large pasture that is, in some places, heavily grazed by cattle. Due to the animals and strong rainfall, erosion is a common phenomenon on the steep slopes. The resulting bare soil is soon colonized by a number of *Asplenium* species, including *A. adiantum-nigrum*, *A. azoricum*, *A. marinum*, *A. obovatum* ssp. *lanceolatum*, *A. onopteris*, and *A. scolopendrium*. Similar conditions, with periodic disturbance of soil banks, previously has been shown to promote hybrid formation between *Asplenium* species (Jee, 1994).

Discussion and Conclusions

The endemic *Asplenium azoricum* is widely distributed throughout the Azorean archipelago and is the most common representative of the *A. trichomanes* group on these islands. As it is able to survive even in dense plantations of invasive species, it cannot be considered endangered, although on those islands where it is very rare, e.g., Corvo, its few localities should be afforded protection. It is one of several fern species endemic to the Macaronesian region that almost certainly have evolved in the region but are not now sympatric with both their putative parents. It is hoped that ongoing phylogenetic studies will elucidate the relationship of *A. azoricum* and *A. trichomanes* ssp. *coriaceifolium* to the, as yet unknown, non-European parent they may share.

The merit of attempting to protect sterile fern hybrids is contentious, especially when resources are limited and many other endemic species are under threat, as in the Azores. Often the formation of the hybrids has been dependant on disturbance to sites through human activities. Arguably, if conditions and healthy populations of the parental taxa are maintained then it is likely that hybrids will continue to be sporadically produced. However, a strong case can be made for their conservation as unique biological entities with considerable evolutionary potential. Hybridization followed by polyploidy is the most rapid route to the generation of novel species and would seem to be the prevalent mode of speciation within the pteridophyta. These natural hybrids give us a rare opportunity to observe the process of alloploid speciation and the mechanisms and controlling factors behind it.

Acknowledgments

We are grateful to Prof. Dr. J. J. Schneller (Zürich) for permission to use his cytological result in this publication, to Dr. Norman Robson (NHM) for his assistance with the latin descriptions and to the director of the Botanical Museum, Berlin-Dahlem (B) for the loan of specimens. HS thanks the German National Merit Foundation for a PhD grant.

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Phylogenetic Relationships of the Subfamily Taenitidoideae, Pteridaceae

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ABSTRACT.—Thirteen genera are traditionally recognized in the subfamily Taenitidoideae, Pteridaceae. A phylogenetic study of this subfamily, based on both morphological and molecular data, was performed using an exemplar approach. Representatives of the following genera were included in the analyses: *Jamesonia*, *Eriosorus*, *Pterozonium*, *Syngramma*, *Taenitis*, *Austrogramme*, *Pityrogramma*, *Anogramma*, *Actiniopteris*, *Onychium*, and *Afropteris*. Specimens and DNA samples were not available for *Cerosora* and *Nephopteris*, so they were excluded. Three species were chosen as outgroups: *Pteris multifida*, *P. quadriaurita*, and *Coniogramme fraxinea*, all of which are restricted to the Old World. A robust phylogeny was generated based on 26 morphological characters, 578 base pairs of the plastid gene *rps4* and partial data from the intergenic spacer *rps4-trnS*. The results reject the hypothesis of monophyly of the subfamily as presented by Tryon *et al.* (1990). However, the results support the monophyly of a well-supported clade consisting of *Jamesonia*, *Eriosorus*, *Pterozonium*, *Austrogramme*, *Syngramma*, *Taenitis*, *Pityrogramma*, and *Anogramma*. The New World genera *Jamesonia* and *Eriosorus* form a monophyletic group, and *Pterozonium* is more closely related to the Old World genera, *Austrogramme*, *Syngramma*, and *Taenitis*.

Although, ferns are the second most species-rich group of land plants, they have been relatively understudied compared to the largest group, the flowering plants. Several comprehensive studies on pteridophytes have looked at morphological and/or molecular characters from a phylogenetic perspective (Hasebe *et al.*, 1993, 1994, 1995; Pryer *et al.*, 1995; Schneider, 1996; Wolf *et al.*, 1998; Pryer *et al.*, 2001). While the majority of studies have focused on establishing higher-level relationships (Wolf *et al.*, 1998; Pryer *et al.*, 2001), an increasing number of studies have looked closely at lower-level relationships (Conant *et al.*, 1995; Haufler *et al.*, 1995; Gastony and Rollo, 1995; Pryer, 1999; Gastony and Johnson, 2001; Smith and Cranfill, 2002; Ranker *et al.*, 2003). Such phylogenetic studies at lower taxonomic levels are sorely needed to facilitate understanding of evolutionary processes of diversification and biogeographic patterns among pteridophytes.

The Pteridaceae is a large and diverse family of homosporous ferns with a nearly worldwide distribution (Tryon *et al.*, 1990). The family comprises 34 genera that are mostly restricted to the New World (Tryon *et al.*, 1990). A considerable number of species are found in exposed and rocky environments, although some members of the family are found in a diverse array of mesic habitats (Tryon *et al.*, 1990). Phylogenetic relationships within the Pteridaceae

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are poorly understood. Of the six subfamilies recognized in the family (Tryon *et al.*, 1990), only the Cheilanthoideae has been extensively studied from a phylogenetic perspective, using plastid *rbcL* and nuclear ITS nucleotide sequences (Gastony and Rollo, 1995, 1998). Some members of subfamily Taenitidoideae were included in a larger phylogenetic analysis based on *rbcL* nucleotide sequences (Gastony and Johnson, 2001; Nakazato and Gastony 2003), and more studies are needed to understand the phylogenetic relationships amongst its members. Phylogenetic relationships within and among the other five subfamilies are yet to be resolved.

Subfamily Taenitidoideae is difficult to circumscribe morphologically. Some of the most distinctive and diagnostic morphological characters are not consistent across all members of the group. In general, the sporangia are borne along the veins in exindusiate soral lines, or on an inframarginal commissural vein, but in some genera the sporangia are borne at the leaf margin and are protected by a false indusium. Most genera in the Taenitidoideae have paraphyses associated with the sporangia, but some genera completely lack paraphyses.

According to the most recent taxonomic review (Tryon *et al.*, 1990) thirteen genera belong to subfamily Taenitidoideae, which has a worldwide distribution. *Jamesonia*, *Eriosorus*, *Pterozonium*, and *Nephopteris* are primarily neotropical (Tryon *et al.*, 1990). *Pityrogramma* and *Anogramma* are mostly restricted to the Neotropics, but the former extends to Madagascar, and the latter is subcosmopolitan. *Actiniopteris* is primarily African, and *Syngramma*, *Taenitis*, and *Austrogramme* are centered in southeastern Asia. *Afropteris* is distributed in tropical West Africa and the Seychelles. *Onychium* is found from northeastern Africa, from Iran eastward to China, as well as in New Guinea. *Cerosora* is a native of Borneo, Sumatra, and the Himalayas. The traditionally recognized members of subfamily Taenitidoideae exhibit a diversity of habitats, ranging from moist and sheltered to dry and open, and from terrestrial to rupestral. Some species live in forests, in the understory growing along streams, and a few species are rheophytes.

Historically the Old World genera *Syngramma*, *Taenitis*, and *Austrogramme* have been considered to make up a natural group based on a number of shared morphological characters such as disposition of sporangia and paraphyses (Copeland, 1947; Holttum, 1959, 1960, 1968, 1975; Walker, 1968; Hennipman, 1975). The New World genera *Jamesonia*, *Eriosorus*, and *Pterozonium* have also been considered closely related to each other based on venation, blade indument, and spores (A. Tryon, 1962). A. Tryon (1970) postulated that *Eriosorus* represents the least advanced group among the mainly American genera, and that its relationship to *Pterozonium* is not as close and is without a clear lineal derivation. She also suggested that *Jamesonia* and *Eriosorus* are closely related, and that *Jamesonia* is derived from more than one element in *Eriosorus*. A. Tryon (1962) pointed out morphological similarities shared between the Old and New World genera of the Taenitidoideae, characters that suggested a close phylogenetic relationship. Recent studies have suggested that the Old World genera of the Taenitidoideae and *Pterozonium* originated in

southern Gondwana region before South America and Antarctica-Australia separated during the Lower Cretaceous (Schneider, 2001). Furthermore, *Pityrogramma* and *Anogramma* have been hypothesized to be closely related to each other based on lamina architecture, sorus type, and spores (A. Tryon, 1962).

The general aim of this study was to elucidate phylogenetic relationships within the subfamily Taenitidoideae based on both morphological and molecular data. As a working hypothesis, monophyly of the Taenitidoideae as presented by Tryon *et al.* (1990) was assumed. A second aim was to test various hypotheses of relationships within and among the Old and New World genera of the subfamily. A third aim was to establish the closest relatives of *Jamesonia* and *Eriosorus*.

Material and Methods

Specimens Examined.—Using the exemplar approach, 20 species were chosen to represent eleven of the thirteen genera currently recognized in the subfamily. Vouchers and DNA samples were not available for *Cerosora* and the monotypic genus *Nephopteris*, so they were excluded. A complete list of the taxa used in this study is presented in Table 1, which refers to vouchers that were used to generate sequence data for *rps4* as well as morphological characters. Morphological characters were also corroborated with other specimens housed at the University Herbarium, University of California, Berkeley (UC). Most of the DNA vouchers included in this study are housed at UC. Two specimens are at the Nationaal Herbarium Nederland, Leiden (L), and one specimen is at Institut für Systematische Botanik der Universität Zürich (Z).

A multiple outgroup approach was used to resolve plesiomorphic characters within the ingroup (Maddison *et al.*, 1984). *Pteris multifida* and *P. quadriaurita* from the subfamily Pteridoideae were included based on broader-scale previous phylogenetic studies (Hasebe *et al.*, 1995; Pryer *et al.*, 1995). *Coniogramme fraxinea* was also included as a more distantly related outgroup (Hasebe *et al.*, 1995; Pryer *et al.*, 1995; Gastony and Rollo, 1995; Gastony and Johnson, 2001). *Coniogramme* was initially placed in the cheilanthoids by Tryon *et al.* (1990) but subsequently shown to be the sister to other traditional Pteridaceae plus Vittariaceae (Hasebe *et al.*, 1995; Gastony and Rollo, 1998; Nakazato and Gastony 2003). All three outgroups are restricted to the Old World.

Morphological Character Analysis.—The following criteria were considered when selecting morphological characters for this study: 1) characters should exhibit greater degree of variability among OTUs than within, thus providing discrete character states; 2) characters should lack variability due to ecophenotypic factors; 3) characters should be independent of each other (Wiley, 1981); and 4) there should be a good basis for hypothesizing homology across the study group.

As a first approach, literature on traditional classifications of genera in the Taenitidoideae, and previously published morphological descriptions were

TABLE 1. Species used as a source for DNA and *rps4* sequence data for this study. Most material was preserved in silica gel, two were herbarium specimens, and a few were fresh material.

Genus Species	Collector/Source/ Herbarium	Geographic origin	Accession number
Actiniopteris australis (L.f.) Link	Sánchez-Baracaldo 360 (UC)	Unknown, native of Africa	AF321693
Afropteris barklyae (Baker)	Kramer 11086 (Z)	Seychelles Islands	AF544984
Anogramma chaerophylla (Desv.) Link	Sánchez-Baracaldo 361 (UC)	Unknown	AY357705
Anogramma guatemalensis (Domin) C. Chr.	Smith 2586 (UC)	Costa Rica	AF321699
Austrogramme decipiens (Mett.) Hennipman	van der Werff 16114 (UC)	New Caledonia	AF321702
Austrogramme marginata (Mett.) E. Fourn.	D. Hodel 1454 (UC)	New Caledonia	AY357704
Coniogramme fraxinea (D. Don) Fée ex Diels	UC Bot. Gard 58.0375 (UC)	Java	AF321696
Eriosorus flexuosus Copel.	Sánchez-Baracaldo 215 (UC)	Colombia, Cundinamarca	AF321710
Eriosorus insignis (Kuhn) A. F. Tryon	A. Salino 3010 (UC)	Brazil, Minas Gerais	AF321708
Eriosorus rufescens (Fée) A. F. Tryon	Sánchez-Baracaldo 268 (UC)	Colombia, Antioquia	AF321719
Jamesonia alstonii A. F. Tryon	Sánchez-Baracaldo 246 (UC)	Colombia, Cocuy	AF321747
Jamesonia imbricata (Sw.) Hook. & Grev.	Sánchez-Baracaldo 252 (UC)	Colombia, Guantiva	AF321756
Onychium japonicum (Thunb.) Kunze	B. Ornduff 10278 (UC)	China, Yunnan	AF321697
Pityrogramma austroamericana Domin	UC Bot. Gard. 98.0063 (UC)	Unknown, native of Neotropics	AF321698
Pteris multifida Poir.	UC Bot. Gard. 80059 (UC)	Unknown, native of Old World	AF321695
Pteris quadriaurita Retz.	UC Bot. Gard. 67.1645 (UC)	Unknown, pantropical	AF321694
Pterozonium cyclosorum A. C. Sm.	Brewer et al. 1006 (UC)	Venezuela, Bolívar	AF321703
Pterozonium reniforme (Mart.) Fée	Brewer et al. 1005 (UC)	Venezuela, Amazonas	AF321704
Syngramma quinata (Hook.) Carr.	M. Kessler 2273 (L)	Borneo, West Kalimantan	AF321701
Taenitis interrupta Hook. et Grev.	H. Schneider 1031 (L)	Borneo, Sarawak	AF321700

reviewed (Ching, 1934; Pichi Sermolli, 1962; A. Tryon, 1962; R. Tryon, 1962; Lellinger, 1967; Holttum, 1968; Atkinson, 1970; Holttum, 1970, 1975, Tryon and Lugardon, 1991). From this list, those characters that met the above criteria were selected and modified. In addition, some new characters not previously considered were examined and included. In the present study, non-applicable characters were coded as missing data, an approach previously discussed in

TABLE 2. Morphological data matrix for the cladistic analysis of subfamily Taenitidoideae, Pteridaceae. See Appendix 1 for characters and characters states.

Taxa	Character number																									
										1										2						
	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6
Jamesonia imbricata	1	0	1	1	0	1	1	1	1	1	0	0	1	1	2	0	1	1	2	0	0	1	?	?	0	1
Jamesonia alstonii	1	0	1	1	0	1	1	1	1	1	0	0	1	1	2	0	1	1	2	0	0	1	?	?	0	1
Eriosorus insignis	1	0	0	1	0	1	1	1	1	1	0	0	1	0	0	0	0	1	2	0	0	1	?	?	0	1
Eriosorus rufescens	1	0	0	1	0	1	1	1	1	1	0	0	1	0	0&1	0	0	1	2	0	0	1	?	?	0	1
Eriosorus flexuosus	1	0	0	1	0	1	1	1	1	1	0	0	0	0	0	0&1	1	0	1	0	0	1	?	?	0	1
Pterozonium reniforme	1	0	0	2	0	0	1	1	0	1	0	1	2	0	0	1	1	0	1	0	1	1	?	?	0	1
Pterozonium cyclosorum	1	0	0	2	0	0	1	1	1	1	0	1	2	0	0	1	1	0	2	0	1	1	?	?	0	1
Austrogramme decipiens	1	1	?	2	2	0	0	1	1	1	0	1	1	0	0	1	1	0	0	0	0	1	?	?	0	0
Austrogramme marginata	1	1	?	2	2	0	0	1	1	1	0	1	2	0	0	1	1	0	0	0	0	1	?	?	0	0
Syngramma quinata	1	1	?	2	2	0	0	1	1	1	0	1	1	0	0	1	1	0	0	0	0	1	?	?	0	1
Taenitis interrupta	1	0	0	2	2	0	0	1	1	1	0	1	1	0	0	1	1	0	0	0	0	1	?	?	0	1
Anogramma guatemalensis	1	0	2	0	0	1	0	1	1	1	1	?	0	0	0	1	1	0	0	0	1	0	3	0	1	?
Anogramma chaerophylla	1	0	2	0	0	1	0	1	1	1	1	?	0	0	0	1	1	0	0	0	1	0	3	0	1	?
Pityrogramma austroamericana	1	0	2	0	0	1	0	1	0	1	1	?	0	0	0	1	1	0	0	0	1	0	2	0	1	?
Onychium japonicum	1	0	1	0	1	?	0	0	0	0	1	?	0	0	0	1	1	0	0	1	1	0	2	0	1	?
Actiniopteris australis	1	0	1	0	1	1	0	0	1	0	1	?	3	0	0	1	1	0	0	1	1	0	1	0	1	?
Afropteris barklyae	0	0	0	2	2	?	0	0	1	0	1	?	0	0	0	1	1	0	0	1	1	0	0	0	1	?
Coniogramme fraxinea	1	1	?	3	1	0	0	1	1	1	1	?	1	0	0	1	1	0	0	0	0	0	0	2	1	1	?
Pteris multifida	0	0	0	0	0	?	0	0	1	0	0	?	0	0	0	1	1	0	0	0	0	0	0	0	0	1	?
Pteris quadriaurita	0	0	0	0	0	?	0	0	1	0	0	?	0	0	0	1	1	0	0	0	0	0	0	0	0	1	?

Maddison (1993). Non-applicable characters occur when taxa lack the structure in question, for instance, in the present morphological data set, color of scales was scored only for *Anogramma*, *Pityrogramma*, *Onychium*, *Actiniopteris*, *Afropteris*, *Pteris*, and *Coniogramme* because the other genera lack scales.

Some morphological characters were sought from cleared leaves mounted on slides (Arnott, 1959) from each exemplar. Two to three slides were mounted per exemplar. A total of 26 characters were included in the analyses. The data matrix with the characters and character states is shown in Table 2, and a detailed description of the characters used is presented in Appendix 1.

MOLECULAR CHARACTERS.—A list of the taxa studied and their respective vouchers is presented in Table 1. Total genomic DNA was extracted using DNeasy Plant Mini Kits (Qiagen, Chatsworth, CA), following the manufacturer's protocol. The amplicons of *rps4* were amplified by polymerase chain reaction (PCR), using the forward primer rps5 and reverse primer trnS (Souza-Chies *et al.*, 1997). PCR reaction mixtures each contained 0.5 units of AmpliTaq Gold polymerase (PE Applied Biosystems), 5 μL of the supplied 10x Buffer II (2.5 mM $MgCl_2$), 0.1 mM of each dNTP, 2.5 mM of each primer, ~50 ng of total genomic DNA and purified water to volume.

PCR cycles (Perkin Elmer GeneAmp PCR System 9600 thermocycler) were programmed as follows: an initial hot start of 95°C for 10 min to activate the AmpliTaq Gold polymerase, 40 cycles (94°C for 30 s, 60°C for 45 s, and 72°C for

2 min), and a 7 min final extension step at 72°C. PCR products were visualized with ethidium bromide on 1% agarose gels which were run in 1 X Tris-borate/EDTA electrophoresis buffer (pH 7.8). Amplicons were purified with the QIAquick PCR purification kit (Qiagen, Chatsworth, CA) following the manufacturer's protocol, and then processed by cycle sequencing and BigDye-terminator chemistry (PE Applied Biosystems) on an ABI model 377 automated fluorescent sequencer in the Molecular Phylogenetics Laboratory at the University of California, Berkeley.

Sequence files were edited by visual inspection of electropherograms, and mutations or changes were verified using the program Sequence Navigator (PE Applied Biosystems). Alignments were performed by eye in a nexus file. The final aligned data matrix consisted of 993 characters; 578 from the *rps4* coding region, and 415 from the intergenic spacer *rps4–trnS*. For *Eriosorus* and *Jamesonia*, 413 bp from the intergenic spacer *rps4–trnS* were included; twelve distinct shared insertion/deletion regions were recognized in the final alignment and each region was coded as a single binary character for the maximum parsimony analyses. For *Pterozonium*, *Austrogramme*, *Syngramma* and *Taenitis*, 252 bp were included from the intergenic spacer *rps4–trnS*; nine distinct shared insertion/deletion regions were recognized in the final alignment and each region was coded as a single binary character for the maximum parsimony analyses. The whole intergenic spacer *rps4–trnS* region was excluded due to ambiguity in the alignment for the following taxa: *Anogramma chaerophylla*, *A. guatemalensis*, *Pityrogramma austroamericana*, *Onychium japonicum*, *Actiniopteris australis*, *Afropteris barklyae*, and the three outgroups.

PHYLOGENETIC ANALYSIS.—The morphological data set was compiled using MacClade 4.0 (Maddison and Maddison, 2000). All maximum parsimony and bootstrap analyses were run in PAUP* 4.0b10 (PPC; Swofford, 1999). Multistate characters were unordered, and uninformative characters were excluded in all analyses. For each analysis, maximum parsimony analyses were performed, and stepwise addition searches were conducted with the following specifications: 1000 random additions, tree-bisection-reconnection (TBR) branch-swapping, and MULPARS. Equally most parsimonious trees were summarized using a strict consensus tree. Bootstrap values were calculated (Felsenstein, 1985; Sanderson, 1989; Hillis and Bull, 1993) to provide a measurement of support. Bootstrapping of all data sets used 1000 replicates, with 100 random addition starting trees implemented for each replicate, TBR branch swapping, and MULPARS. The three analyses that were carried out in this study are as follows: 1) morphological data; 2) molecular data; and 3) both morphological and molecular data.

RESULTS

The morphological data set included 26 characters; of these, 25 were parsimony-informative and one was autapomorphic. The molecular data set

included 993 characters; of these, 309 sites were variable of which 186 were parsimony-informative and 123 were parsimony-uninformative.

MORPHOLOGICAL ANALYSIS.—A single most parsimonious tree (Fig. 1) was found at 50 steps (CI = 0.70; RI = 0.86). Only bootstrap values higher than 50% are reported. Results of this analysis weakly supported the monophyly of the Neotropical genera, *Jamesonia*, *Eriosorus*, and *Pterozonium*, plus the Old World genera *Austrogramme*, *Syngramma*, and *Taenitis*. Within this clade, *Pterozonium* was basal to *Eriosorus* and *Jamesonia*. *Eriosorus* and *Jamesonia* together formed a monophyletic group. *Eriosorus* appeared to be paraphyletic containing a monophyletic *Jamesonia*. *Onychium japonicum*, and *Actiniopteris australis* formed a monophyletic group within a weakly supported clade including also *Afropteris barklyae*, *Pteris multifida*, and *P. quadriaurita*.

MOLECULAR AND COMBINED DATA ANALYSES.—The parsimony analysis of molecular characters generated a total of two equally most parsimonious trees found at 481 steps (CI = 0.76; RI = 0.83); the strict consensus is shown in Fig. 2. The parsimony analysis of morphological and molecular characters combined resulted in a total of four equally most parsimonious trees found at 539 steps (CI = 0.75; RI = 0.82); the strict consensus is shown in Fig. 3. Only bootstrap values higher than 50% are reported.

Results for the molecular data and the combined data sets are described together because the strict consensus topologies of both analyses agreed in almost every aspect (Figs. 2, 3), except for an unresolved node of the clade containing *Afropteris barklyae*, *Pteris multifida*, and *P. quadriaurita* (Fig. 3). Both analyses support the monophyly of clades containing *Austrogramme*, *Syngramma*, *Taenitis*, and *Pterozonium*, as well as the Neotropical clade of *Jamesonia* and *Eriosorus*. *Eriosorus* itself appears to be paraphyletic and includes a monophyletic *Jamesonia*. In both analyses, *Anogramma* and *Pityrogramma* form a monophyletic group, which is sister to the monophyletic group that includes *Jamesonia*, *Eriosorus*, *Pterozonium*, *Austrogramme*, *Syngramma*, and *Taenitis*, with high bootstrap support.

Pteris multifida, *P. quadriaurita*, and *Afropteris barklyae* formed a highly supported monophyletic group in both analyses (Figs. 2, 3). In the molecular analysis, *Afropteris barklyae* is nested within *Pteris* (Fig. 2), while in the combined analysis the relationship of *Afropteris barklyae*, *Pteris multifida* and *P. quadriaurita* is unresolved (Fig. 3). *Onychium* and *Actiniopteris* form a well-supported monophyletic group, that appears sister to the *Afropteris-Pteris* clade (Figs. 2, 3).

Outgroups.—Even if all analyses were rooted with both species of *Pteris* and *Coniogramme fraxinea*, *Pteris multifida* and *P. quadriaurita* were consistently nested with *Afropteris* (Figs. 1–3). In the morphological analysis, *P. multifida* and *P. quadriaurita* form a monophyletic group that is sister to *A. barklyae*, although with very low bootstrap support (Fig. 1). The relationship of the *Afropteris-Pteris* clade to *Onychium japonicum* and *Actiniopteris australis* is weakly supported (Figs. 1–3). Both molecular and combined data sets strongly

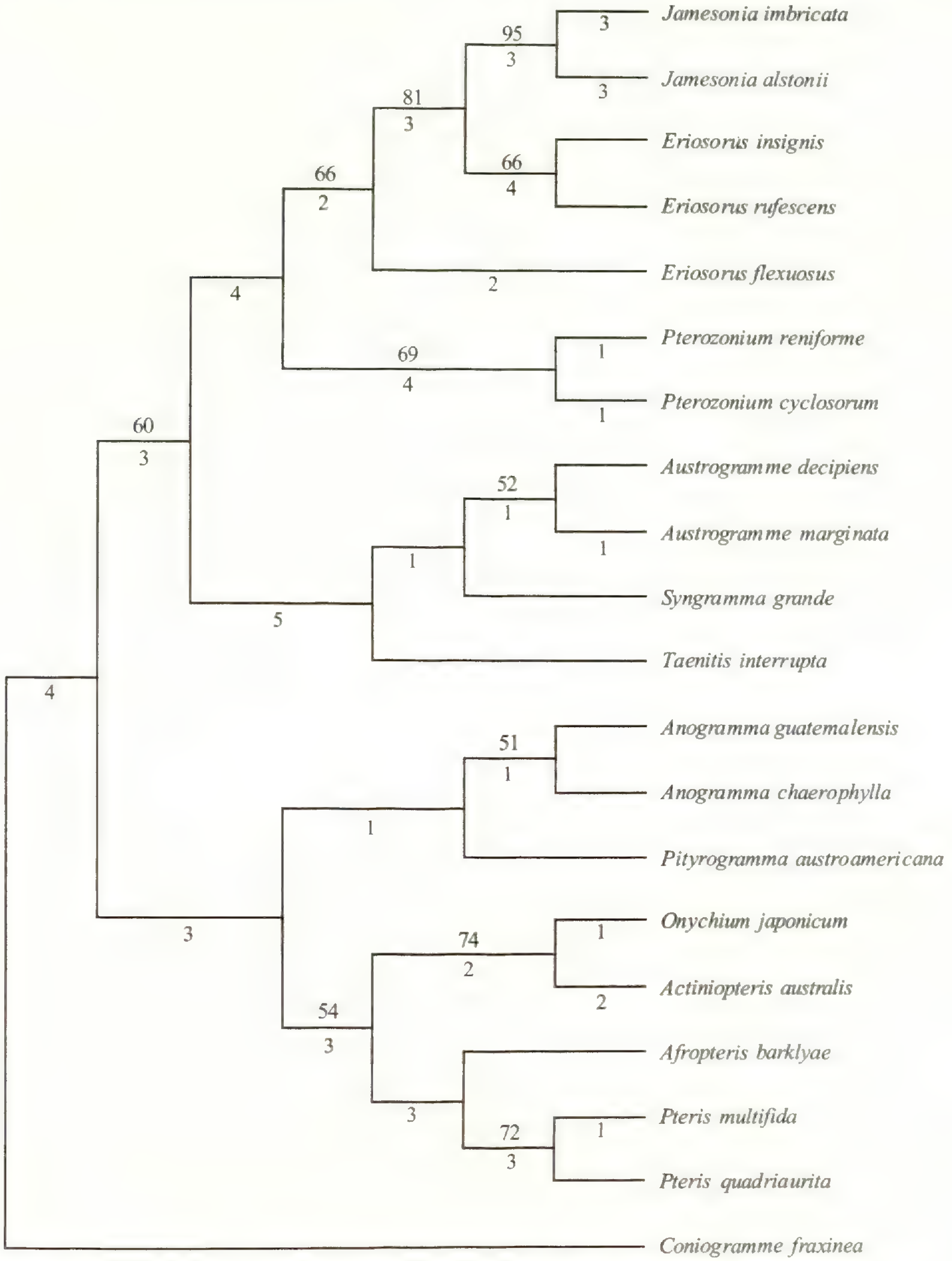

FIG. 1. Parsimony analysis of morphological data set. Single most parsimonious tree of 50 steps (CI = 0.70; RI = 0.86). Numbers above branches indicate bootstrap percentage values based on 1000 replicates of 100 random addition sequence replicates each. Numbers of character state changes per branch are indicated below the lines. The tree was rooted using the outgroups *Pteris multifida* and *P. quadriaurita* from subfamily Pteridoideae, and a more distantly related member, *Coniogramme fraxinea*, as explained in the text.

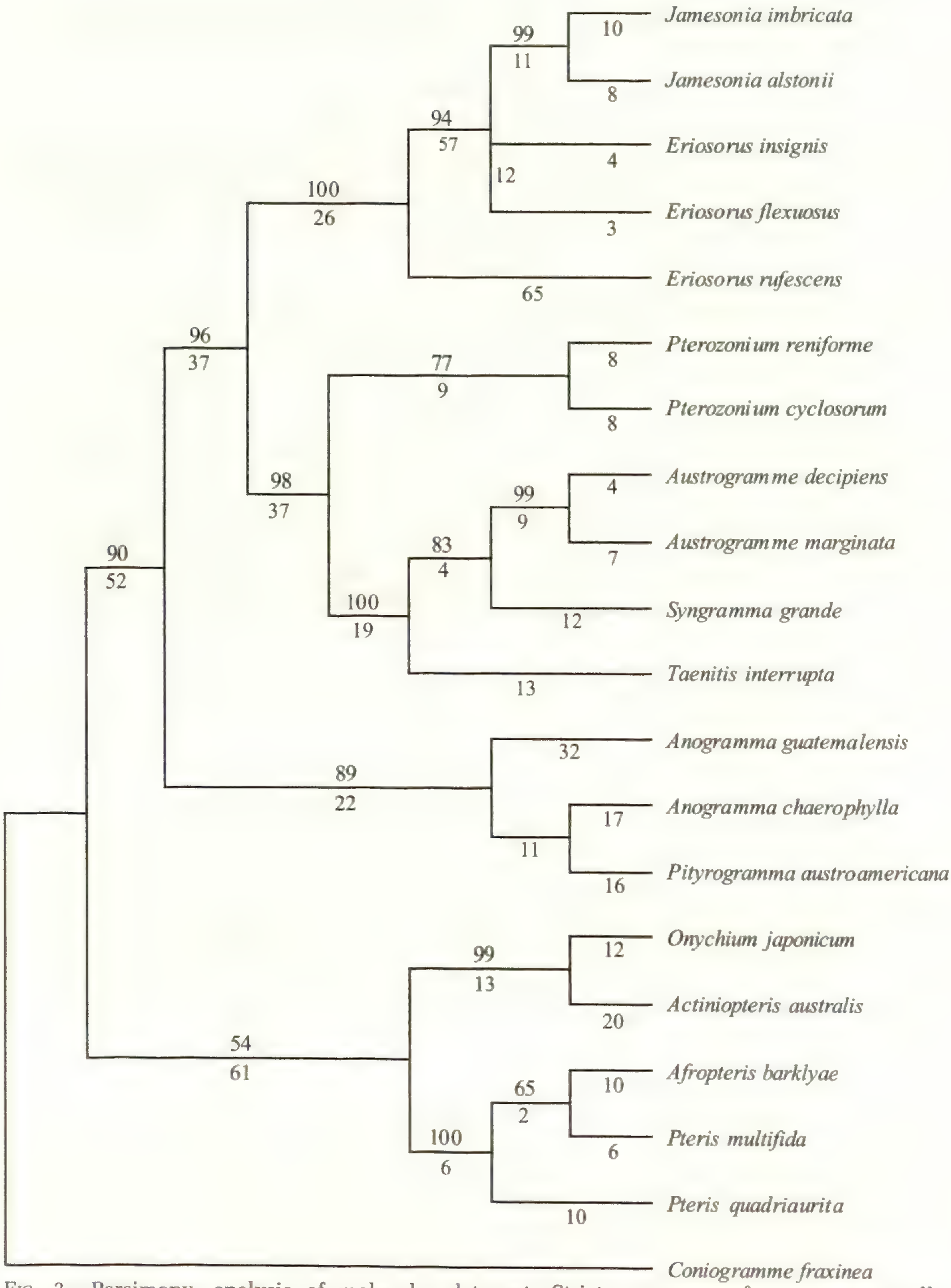

FIG. 2. Parsimony analysis of molecular data set. Strict consensus of two most equally parsimonious trees at 418 steps (CI = 0.76; RI = 0.83). Numbers above branches indicate bootstrap percentage values based on 1000 replicates of 100 random addition sequence replicates each. Numbers of character state changes per branch are indicated below the lines. Trees were rooted using the outgroups *Pteris multifida* and *P. quadriaurita* from subfamily Pteridoideae, and a more distantly related member, *Coniogramme fraxinea*, as explained in the text.

support the relationship between *Afropteris barklyae*, *Pteris multifida*, and *P. quadriaurita* (Figs. 2, 3).

Discussion

PHYLOGENETIC RELATIONSHIPS.—Bootstrap values robustly support the topologies generated by the analyses of the molecular data alone and the combined data sets, but are weak in the analysis of the morphological data set. Clade support values for the combined data sets are slightly higher than for the molecular data set alone. The outgroup rooting employed rejects the hypothesis of monophyly of subfamily Taenitidoideae as defined by Tryon *et al.* (1990). In this study, *Pteris* appears to be closely related to a member of the ingroup (e.g. *Afropteris*) suggesting that it would be more appropriately classified with the pteridois as initially proposed by Tryon and Tryon (1982). However, all analyses agree on the monophyly of a highly supported clade including: *Jamesonia*, *Eriosorus*, *Pterozonium*, *Austrogramme*, *Syngramma*, *Taenitis*, *Anogramma*, and *Pityrogramma* (Figs. 1–3). The most robust analyses, the molecular and combined data sets, recover a well supported monophyletic group including: *Jamesonia*, *Eriosorus*, *Pterozonium*, *Austrogramme*, *Syngramma*, *Taenitis*, *Anogramma*, and *Pityrogramma* (Figs. 2, 3). In addition, all analyses performed in this study indicate that *Jamesonia* and *Eriosorus* form a monophyletic group (Figs. 1–3).

Based on the analysis of morphological characters alone, *Pterozonium* is sister to the clade consisting of *Jamesonia* and *Eriosorus* (Fig. 1); this clade is defined here by acropetal (outward) sporangial maturation (character 7, Appendix 1) shared by these three genera. In contrast, the most robust analyses based on DNA sequences alone and the combined data sets suggest that the New World genus *Pterozonium* is more closely related to three Old World genera, *Austrogramme*, *Syngramma*, and *Taenitis* (Figs. 2, 3); a number of morphological characters states are shared by this clade, e.g., spore ornamentation, sporangial disposition, and paraphyses disposition (characters 4, 7 and 12 respectively, Appendix 1).

PREVIOUS HYPOTHESES OF RELATIONSHIPS.—The topologies presented in this study prompt discussion of several previously proposed phylogenetic hypotheses. The Old World genus *Austrogramme* is closely related to *Syngramma* and *Taenitis*, as postulated by Walker (1968). In all analyses, *Syngramma* and *Taenitis* are closely related, as hypothesized by Copeland (1947) and Holttum (1960, 1975), with *Taenitis* being basal to *Syngramma* and *Austrogramme* (Figs. 2, 3).

Although, A. Tryon (1962) postulated that the neotropical genera *Pterozonium*, *Eriosorus*, and *Jamesonia* constitute a natural group, she later (1970) stated about *Eriosorus* that, "the relationship to *Pterozonium* is not as close and is without clear linear derivation." The results in this study (Figs. 2, 3) suggest that *Pterozonium* is actually more closely related to the Old World genera *Austrogramme*, *Syngramma*, and *Taenitis* (Figs. 2, 3) as proposed by Schneider

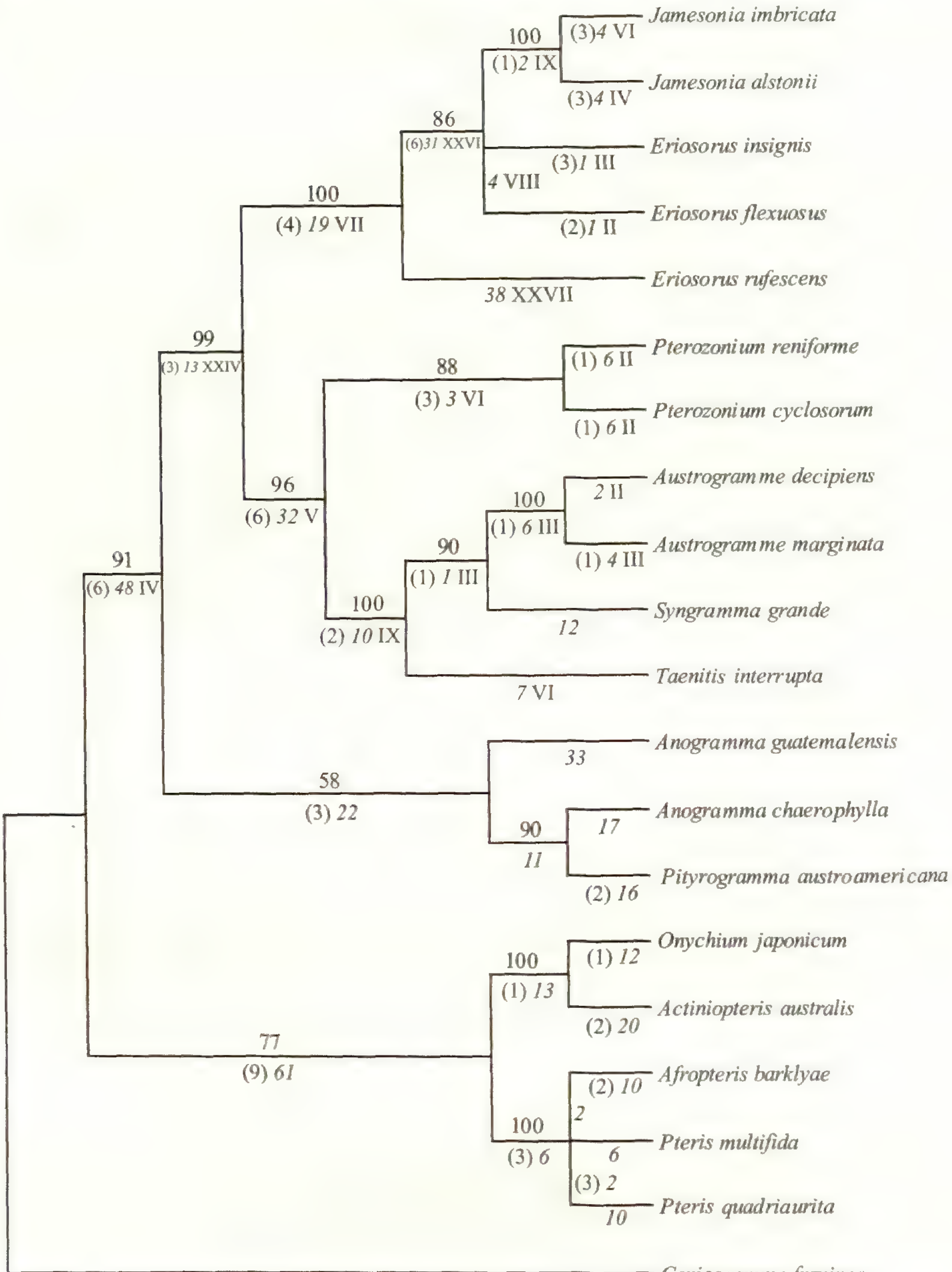

FIG. 3. Parsimony analysis of morphological and molecular combined data sets. Strict consensus of four equally most parsimonious trees at 539 steps (CI = 0.75; RI = 0.82). Numbers above branches indicate bootstrap percentage values based on 1000 replicates of 100 random addition sequence replicates each. Numbers of morphological steps per branch are indicated in parentheses. Numbers of supporting molecular characters per branch, derived from the coding region of *rps4*, are written

(2001). Moreover, A. Tryon (1970) stated that "*Eriosorus* represents the least advanced element among the five, mainly American genera, *Pityrogramma*, *Anogramma*, *Eriosorus*, *Jamesonia* and *Pterozonium*." Evidence presented in this study suggests that *Pityrogramma* and *Anogramma* are sister to the clade containing both New World genera *Jamesonia*, *Eriosorus*, and *Pterozonium*, and certain Old World genera, *Austrogramme*, *Syngramma*, and *Taenitis* (Figs.1–3).

Jamesonia and *Eriosorus* form a monophyletic group, supporting A. Tryon's (1962) hypothesis that both genera might belong to a single genus, in which *Jamesonia* represented the more specialized elements of the larger unit. These hypotheses have been tested and subsequently supported by a more detailed phylogenetic study including 16 species of *Jamesonia* and 14 species of *Eriosorus*, based on a total of 1152 bp from the nuclear External Transcribed Spacer (ETS) of 18S–26S rDNA, and the plastid gene *rps4* and the intergenic spacer *rps4–trnS* (Sánchez-Baracaldo, 2004). Furthermore, it was concluded in that study that neither genus is a natural group: *Jamesonia* is polyphyletic and *Eriosorus* is paraphyletic. *Jamesonia*'s polyphyly had been implicitly hypothesized by A. Tryon (1970): "*Jamesonia* is derived from more than one element in *Eriosorus*."

In all analyses, the species of *Anogramma* and *Pityrogramma* examined here form a monophyletic group as originally postulated by R. M. Tryon (1962). The close relationship between *Anogramma* and *Pityrogramma* species is strongly supported by phylogenetic analyses based on *rbcL* sequence data (Nakazato and Gastony, 2003). They examined more species of *Anogramma* and *Pityrogramma* than here, however, finding that *Anogramma* sensu R. Tryon (1962) is polyphyletic, with *A. osteniana* more closely related to *Eriosorus* and *Jamesonia* then to other species of traditional *Anogramma*. The results of the present study suggest a very strong phylogenetic relationship between the genera *Onychium* and *Actiniopteris*, with a weakly supported relationship to other traditionally recognized taenitidoids, as previously found by Gastony and Johnson (2001), and Nakazato and Gastony (2003). *Afropteris* was treated with the pteridoids (Tryon and Tryon, 1982), before it was reclassified with the subfamily Taenitidoideae (Tryon *et al.*, 1990). Evidence presented here suggests that *Afropteri barklyae* is indeed more closely related to *Pteris multifida*, and *P. quadriaurita* than to the taenitidoids, and suggests that *A. barklyae* would be more accurately classified within the pteridoids as in Tryon and Tryon (1982). Further phylogenetic studies, including broader taxonomic sampling are needed to clarify how this species relates to other

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in *italic* script. Numbers of supporting molecular characters, derived from the intergenic spacer *rps4–trnS*, are indicated in roman script. Trees were rooted using the outgroups *Pteris multifida* and *Pteris quadriaurita* from subfamily Pteridoideae and a more distantly related member, *Coniogramme fraxinea*, as explained in the text.

members of the Pteridaceae. Gastony and Johnson (2001), and Nakazato and Gastony's (2003) work pointed out a close relationship between a clade of *P. fauriei* and *P. cretica* and the taenitidoids, as well as a distant relationship to the outgroup *Coniogramme japonica*. Hypotheses presented in this study are open to further testing with additional taxa. More morphological characters and data from other genes could also help to resolve the history of this group.

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APPENDIX 1

Morphological character list. Characters states follow criteria discussed in text.

1. SPORE SHAPE: electron micrographs are well documented for most genera and many species of ferns. Most taxa included in this study are documented in Tryon and Lugardon (1991). [tetrahedral-deltoid – 0; tetrahedral-globose = 1]

2. EQUATORIAL RINGE: an equatorial flange is defined here as a prominent structure (ring) surrounding a spore at the equatorial plane (Tryon and Lugardon, 1991). [present = 0; absent = 1]

3. NUMBER OF EQUATORIAL RIDGES IN SPORES: Spores in Taenitidoideae exhibit variation in the number of equatorial ridges in different taxa; some taxa completely lack ridges (Tryon and Lugardon, 1991). [one equatorial ridge = 0; two equatorial ridges – 1; three equatorial ridges = 2]

4. SPORE SURFACE: There is great variation in spore ornamentation among members of the Taenitidoideae (Tryon and Lugardon 1991). [extremely verrucose with spines = 0; moderately verrucose – 1; slightly verrucose = 2; smooth = 3]

5. SPORE COLOR: Spore color is a discrete character among members of the Taenitidoideae. This character has been previously used as diagnostic for some genera of the Taenitidoideae (A. Tryon, 1962; 1970). [dark brown = 0; light brown = 1; white = 2]

6. SPORANGIAL DISPOSITION: Exindusiate ferns can exhibit scattered or clustered sporangia along veins. For instance, *Pityrogramma* and *Anogramma* have evenly scattered sporangia in contrast with genera that have clustered sporangia such as *Austrogramme*, *Syngramma*, *Taenitis*, and *Pterozonium*. *Afropteris*, *Onychium*, and *Pteris* were not scored because it was hard to discern the distribution of their sporangia due to their false indusium. [scattered sporangia = 1; clustered sporangia = 2]

7. SPORANGIAL MATURATION: This character refers to sporangial maturation on a fertile leaf. In some fern genera with linear sori, sporangia develop in an outward (acropetal) sequence, along the vein towards the margin. Other genera exhibit mixed sporangial maturation (A. Tryon, 1970). [mixed maturation = 0; acropetal maturation = 1]

8. SPORANGIAL STALK LENGTH: Sporangial stalks vary in length. This character exhibits discrete character states. Taxa with sporangial stalks that were equal to or greater than the capsule length were scored as long. Taxa with sporangial stalks that were extremely short (sessile capsule) or less than half the length of the capsule were scored as sessile to short. Only fully mature sporangia were measured. [long = 0; sessile to short = 1]

9. FARINA: Farina is a waxy-appearing exudate of glands believed to protect young sporangia (Lellinger, 1985). This character can be present in exindusiate and indusiate ferns. [present = 0; absent = 1]

10. INDUSIUM: An indusium is a scale-like structure partially or fully covering and protecting the young sporangia (Lellinger, 1985). In some members of the Pteridaceae, the inrolled lamina edge is modified and called a false indusium. [false indusium = 0; exindusiate = 1]

11. PARAPHYSES: Hairlike structures borne on the soral receptacles or on sporangial stalks or capsules (Lellinger, 1985). Paraphyses are believed to provide protection for young sporangia. [present = 0; absent = 1]

12. PARAPHYSIS ARRANGEMENT: Paraphyses can be densely packed around the sporangia. In contrast, some genera have loose and more relaxed paraphyses associated with their sporangia. [loose=0; densely intermixed=1]

13. FROND DISSECTION: [bipinnate or more = 0; pinnate = 1; simple = 2; pedate = 3]

14. DETERMINATE GROWTH: This character refers to mature fronds bearing sporangia, either maintaining a fiddlehead-like morphology at the tip as adults or not. [determinate = 0; indeterminate = 1]

15. LEAF MARGIN: In some genera, pinna margins are fully extended when mature; in other genera, the pinna margins are more or less incurved, thus protecting the sporangia. In the latter case there is no scale like structure developmentally derived from the leaf margin protecting the sporangia (e.g., false indusium). This character exhibits discrete states. [fully extended = 0; mildly incurved = 1; 1/4 strongly curved = 2]

16. LEAF HAIRS ON ABAXIAL LEAF SURFACE: Hairs are defined as epidermal outgrowths composed of a single elongated cell or a single file of cells. Some species exhibit uniseriate hairs on veins or on abaxial sides of blades. [present = 0 ; absent = 1]

17. STELLATE ARRANGEMENT OF CELLS ON THE LEAF: Cellular configuration of cells associated with only some epidermal hairs on the adaxial side. This character can be observed only with cleared leaves. [present = 0; absent = 1]

18. VEIN ENDINGS WITH RESPECT TO LEAF MARGIN: Strands of vascular tissue can reach or stop before the leaf margin. [veins ending before margin = 0; veins reaching margin = 1]

19. VEIN ENDS: Vascular strands may keep their width or become reduced or enlarged at vein ends. This character is easily observed with cleared leaves. [reduced = 0; same width = 1; enlarged = 2]

20. CELL LENGTH ON ADAXIAL SURFACE OF LEAF: Cells vary in length. In this study, short cells are defined as three to four times longer than wide, and long cells are defined as six to eight times longer as they are wide. This character can be observed only in leaf clearings. [short = 0; long = 1]

21. SHAPE OF CELL WALL ON ADAXIAL SURFACE OF LEAF: Cell wall borders vary among genera; some cell walls are straight while others are sinuous. Among species of *Jamesonia* the degree of sinuosity varies with respect to its position on the leaf (A. Tryon, 1962). However, the cell wall shape, sinuous vs. straight, is a discrete character among genera. The adaxial cells observed for this character were equidistant between veins and margins. This character can be observed only in leaf clearings. [sinuous = 0; straight = 1]

22. SCALES: Scales are defined here as multicellular, bi- to multiseriate epidermal outgrowths (Kubitzki, 1990). In some cases they can also be found on the rhizome, at the base of petioles, and on leaf blades. [present = 0; absent = 1]

23. COLOR OF SCALES: Color of scales is a discrete character among members of this group. [very dark = 0; bicolorous = 1; brown = 2; very pale = 3]

24. SHAPE OF SCALES: Scales exhibit a variety of shapes that seem to be consistent within species but variable across species lines. [elongate = 0; lanceolate = 1]

25. HAIRS ON RHIZOME: Hairs are defined here as uni- to multicellular, uniseriate, epidermal growths (Kubitzki, 1990). [present = 0; absent = 1]

26. HAIR CELLS (ON RHIZOME): Hairs vary in the number of cells at their base. [two cells wide at base = 0; one cell wide at base = 1]

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A Contribution to the Gametophyte Morphology and Development in Several Species of *Thelypteris*, Thelypteridaceae

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Abstract.—A contribution to the study of the gametophyte's morphology and development of some species of *Thelypteris* Schmidel (Thelypteridaceae). The development and morphology of the sexual phase of five species of the Thelypteridaceae family is described and compared. Spores were sown in Thompson medium with agar, germination occurred after 11 days; all spores of the species studied are monolete, ellipsoid with a pleated perine, brown to dark brown; the germination pattern is *Vittaria* type and the prothallial development is *Aspidium* type. Gametangia are of the common type of the leptosporangiate ferns. Sporophytes in *Thelypteris reptans* var. *reptans* and *T. tetragona* appear 90–285 days after sowing; *T. dissimulans*, *T. piedrensis* and *T. oviedoae* did not develop any sporophytes. The sexual phase of these species has many morphological characteristics in common with species of the Old and New Worlds.

The genus *Thelypteris* has *ca* 1000 species distributed in tropical and subtropical regions; the American species have been taxonomically studied by Smith (1971, 1973, 1974, 1980). Approximately 300 species are found in Neotropical areas, *ca* 60 of which are known from Mexico (Smith, 1973, 1974, 1995). Some pteridologists have subdivided *Thelypteris sensu lato* into natural groups as genera, subgenera or sections. Many of the New World taxa can be circumscribed using different combinations of characters and thus be treated as genera. In this paper we treat *Thelypteris* in the broad sense, but recognize several subgenera.

As construed, *Thelypteris* is distinguished by the presence of two vascular bundles in the petiole (*Dryopteris* and other closely related groups have many vascular bundles), acicular hairs on many parts of the blade, spores usually bilateral with a prominent perispore, and a chromosomal base number of 27 to 36 ($x = 40$, 41 in *Dryopteris*, *Athyrium*, and close relatives).

Although the determination of the Thelypteridaceae is based on the sporophyte's morphological characters such as, mainly, the type of indument, for example: bifurcated or stellate hairs (subg. *Goniopteris*); acicular, unicellular or pluricellular hairs (subg. *Goniopteris*), stellate or no furcate hairs (subg. *Macrothelypteris*; subg. *Meniscium* and subg. *Amauropelta*); setose sporangia (subg. *Stegnogramma*); sporangia without setae (subg. *Cyclosorus*, and subg. *Steiropteris*), when doing a follow up of the morphogenetic development, we would expect to find that the gametophyes have the same type of indument, a diagnostic character that would help us to support the segregation at the subgeneric level. Nevertheless, in the studied species we could only observe in the laminar phase, unicellular, capitate hairs, with

a waxy secretion. For this reason we suggest a more detailed study with a larger number of species in order to learn if the indument is a useful character, helpful in separating the species into well defined subgenera, or to join them in infraspecific taxa. Details of the plants' indument provide important taxonomic characters. The indument includes: sessile or pedicellate stellate hair, bifurcate hairs, anchor shaped, fasciculate or hooked hairs; sessile or stipitate glands, setose sporangia or sporangia with stellate hairs or glands. We would expect the plant's gametophytes to share these characteristics.

Although the Thelypteridaceae is very large, its prothallial morphology is poorly known. Its gametophytes can be described as follows: epigeous, chlorophyllous, at maturity cordiform or elongated-cordiform, symmetrical, often with wide wings, fast growing, without a thickened cushion or lacking one, with colorless or light brown rhizoids abundant on the cushion's ventral surface, frequently with unicellular hairs or rarely pluricellular and/or glandular hairs on the margin and on both surfaces. Gametangia are of the advanced leptosporangiate type: the antheridia are characteristically three-celled and dehiscence occurs when the opercular cell detaches; the archegonia are ventral surface, especially on the lower region, the slim neck points towards the meristematic notch (Tryon and Tryon, 1982).

Previous work on *Thelypteris* gametophytes, mainly Asian species include: Schmelzeisen (1933), Momose (1938, 1941), Kachroo (1963), Nayar and Chandra (1963, 1965, 1966), Chandra and Nayar (1968), Devi (1966), Nayar and Devi (1963, 1964), Nayar and Kaur (1969), Mittra and Sen (1981) and Tigerschiöld (1989a, 1989b, 1989c,1990). Details on the prothallia of New World *Thelypteris* are mentioned by Stokey (1960), Atkinson (1971, 1973, 1975a, 1975b), Atkinson and Stokey (1964, 1973). Huckaby and Raghavan (1981b) worked with 16 Jamaican species; Reyes-Jaramillo and Pérez-García (1991) worked with *T. patens* and *T. puberula* var. *puberula*; Pérez-García *et al.* (1994) worked with *T. rhachiflexuosa* and Nester-Hudson *et al.* (1997) worked with *T. ovata* var. *lindheimerii*.

This paper is a contribution to the study of the morphology and development of the gametophytes of *Thelypteris* (*Amauropelta*) *piedrensis* (C. Chr.) Morton, *T.* (*Cyclosorus*) *oviedoae* C. Sánchez & Zavaro, *T.* (*Goniopteris*) *dissimulans* (Maxon & C. Chr. ex C. Chr.) C. F. Reed, *T.* (*Goniopteris*) *reptans* (J.G. Gmelin) Morton var. *reptans* and *T.* (*Goniopteris*) *tetragona* (Sw.) Small.

Materials and Methods

Material for research was collected from the "Jardín Botánico de los Helechos", in Santiago of Cuba and from several different Cuban localities (Table 1). Vouchers are kept at this Herbarium (BSC). Spores were obtained from the fertile leaves of different individuals and leaves with spores were kept in paper envelopes to be released at room temperature. The samples were put through a metallic sieve, with pores 0.074 mm in diameter to remove excess non-spore material. Spores of each species were sown, without sterilization, in 5 Petri dishes, 5 cm in diameter (3 replicates for each species), onto Thompson

TABLE 1. Sites of origin of the taxa under research. Caluff = Manuel García Caluff; Shelton = Gustavo Shelton Serrano, all vouchers have been deposited alive at the BSC (Fern Garden), Santiago de Cuba, Cuba.

Taxa	Vouchers	Locality	Habitat/Altitude
Thelypteris (*Goniopteris*) *dissimulans*	*Caluff & Shelton 4204*	Margins of the "Barao del Banao" river, Santispiritus, Prov. de Santispiritus, Cuba	Gallery forests on limestone rocks, 250 m asl
Thelypteris (*Amauropelta*) *piedrensis*	No date	"Jardín de los Helechos" (Fern Garden)	No date
Thelypteris (*Goniopteris*) *reptans* var. *reptans*	*Caluff & Shelton 2963*	Mogotes of boigne, La Tabla, 3er frente, Prov. de Santiago de Cuba, Cuba	Perennial vegetation, on limestone rocks and slopes, 600 m asl
Thelypteris (*Goniopteris*) *tetragona*	*Caluff 47 A-B*	Altos de Villalón, "La Gran Piedra" mountain range, Prov. Santiago de Cuba, Cuba	Secondary growth, coffee plantations, 500 m asl
Thelypteris (*Cyclosorus*) *oviedoae*	*Caluff s/n*	"Lomas del Solón", las Terrazas, Prov. Pinar del Río, Cuba	Secondary growth on road margins, 600–650 m asl

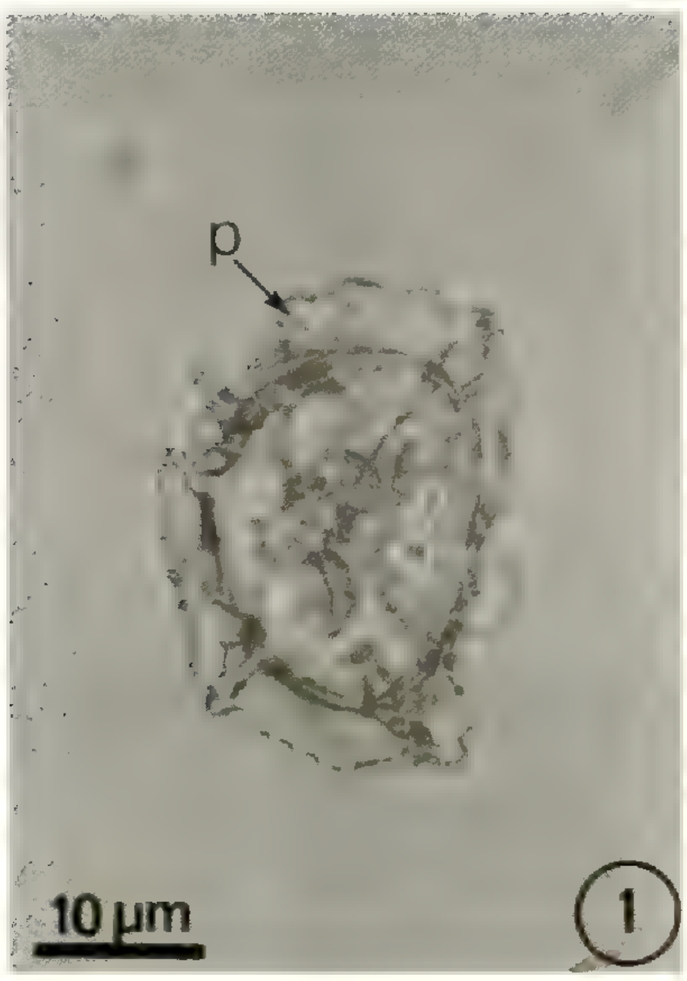
sowing medium (Klekowski, 1969), under aseptic conditions into 5 cm Petri dishes; spores were spread across the medium surface with a brush of scarce bristles. The density of the sown spores varied from 100–150/cm^2.

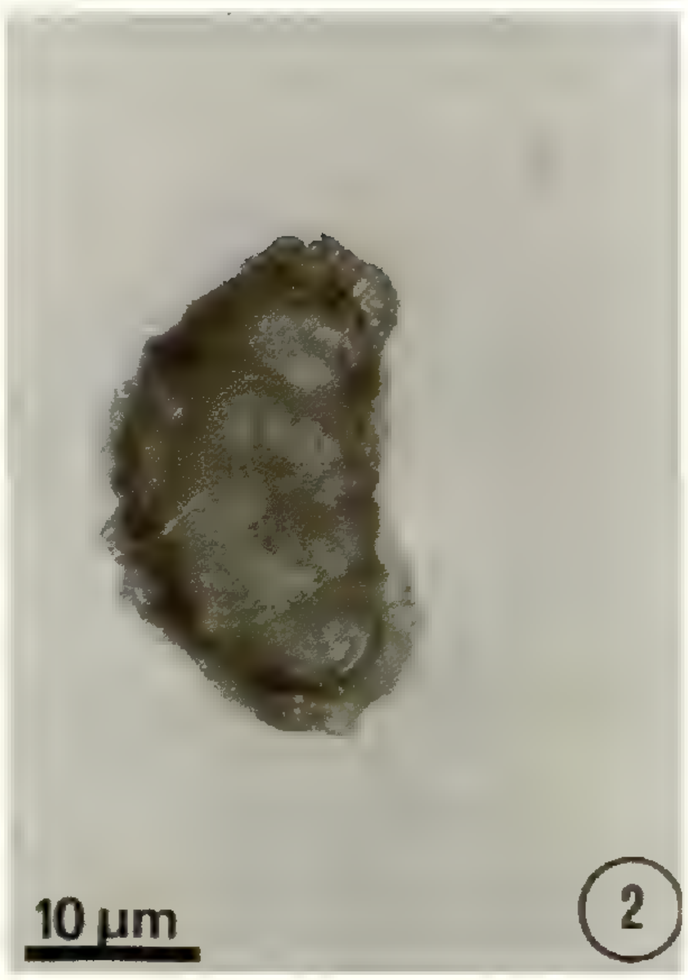
The cultures were kept under lab conditions inside transparent polyethylene bags to avoid contamination and dehydration, under a 12 hr light/12 hr dark photoperiod, with artificial light (Solar 75 Watts, day light) and a 25–28°C temperature. One dish of each taxon was kept in the dark to check for photoblastism. Spores of the five species were also sown separately in soil in 3 small pots, 5 cm in diameter (2 replicates for each species). Photomicrography of all gametophytes was performed on specimens grown on agar in Petri cultures and sporophytes grown in soil samples.

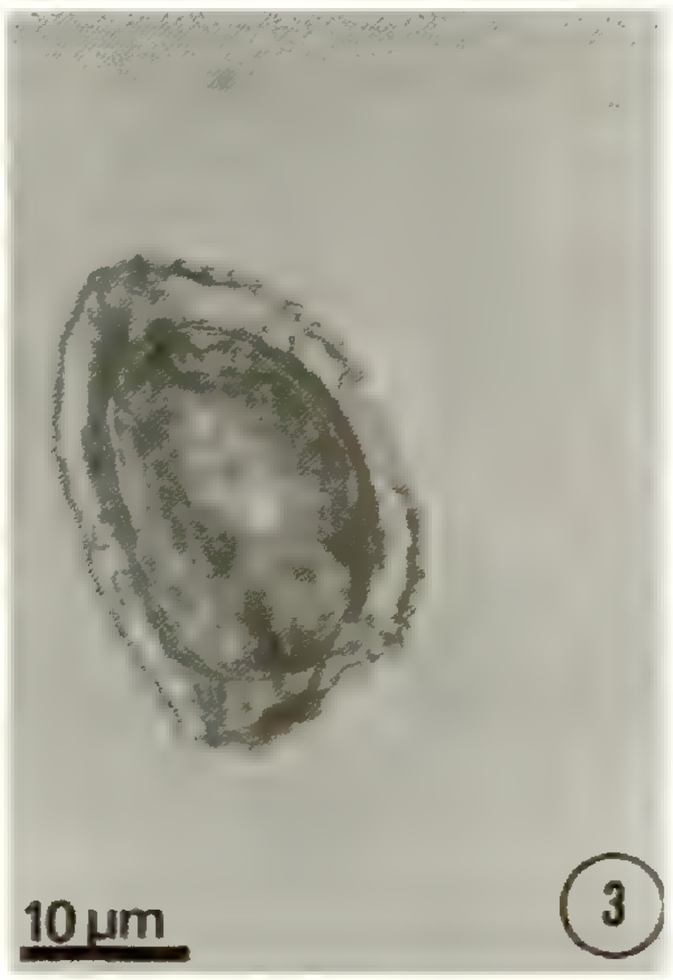
RESULTS

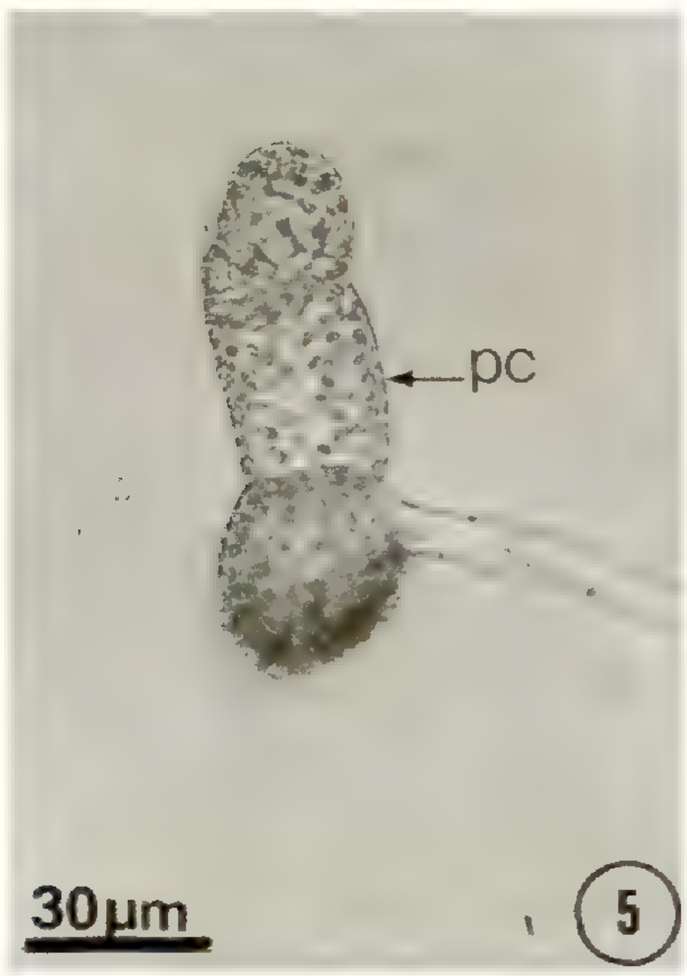
The spores of all species are monolete, with a single leasura and brown to dark-brown. Spores of *T. piedrensis* measure 45 μm × 29 μm (51 μm × 30 μm) 55 μm × 32 μm, of *T. reptans* var. *reptans*, 50 μm × 30 μm (52 μm × 34 μm) 58 μm × 38 μm, of *T. tetragona* 42 μm × 28 μm (44 μm × 29 μm) 47 μm × 32 μm, of *T. dissimulans* 48 μm × 30 μm (49 μm × 33 μm) 50 μm × 35 μm, the perine can be wide or narrow and it has various ornamentations. In *T. dissimulans* (Fig. 1) the perine is quite thick, in *T. piedrensis* (Fig. 2) and *T. oviedoae* it is very thin, and in *T. reptans* var. *reptans* (Fig. 3) and *T. tetragona* it is intermediate in thickness.

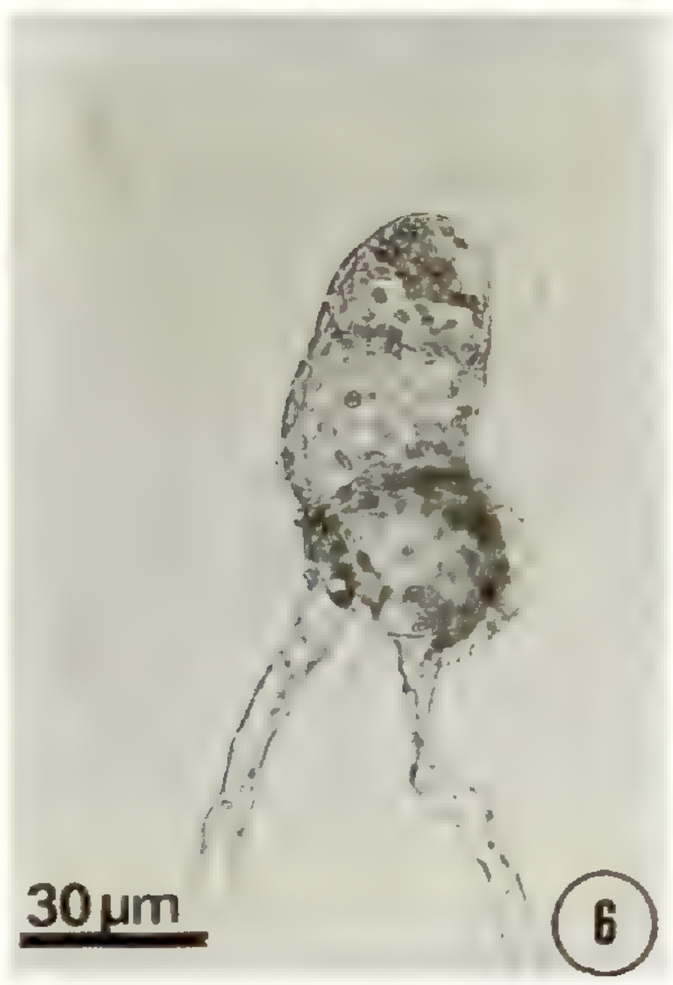
In all species germination began 8–11 days after sowing. Germination is first evidenced by the development of the very long, hyaline rhizoidal cell











FIGS. 1–9. Monolete spores, germination and filamentous phases of *Thelypteris*. Fig. 1–3. Spores. 1. *T. dissimulans*. 2. *T. piedrensis*. 3. *T. reptans* var. *reptans*. 4. Germination in *T. reptans*, (11 days). 5–9. Filamentous phases. 5. *T. dissimulans* (26 days). 6. *T. tetragona* (11 days). 7. *T. piedrensis* (14 days). 8. *T. oviedoae* (15 days). 9. *T. piedrensis* (14 days). p = perine, pc = prothallial cell, r = rhizoid.

containing limited cytoplasm and the prothallial cell which develops inside the spore coat (Fig. 4). This type of germination is the *Vittaria*-type, characterized by the first division giving rise to an initial rhizoid oriented perpendicular to the long axis of the spore. The second division produces an initial prothallial filament at the base of the rhizoid but at right angles to it (Fig. 5).

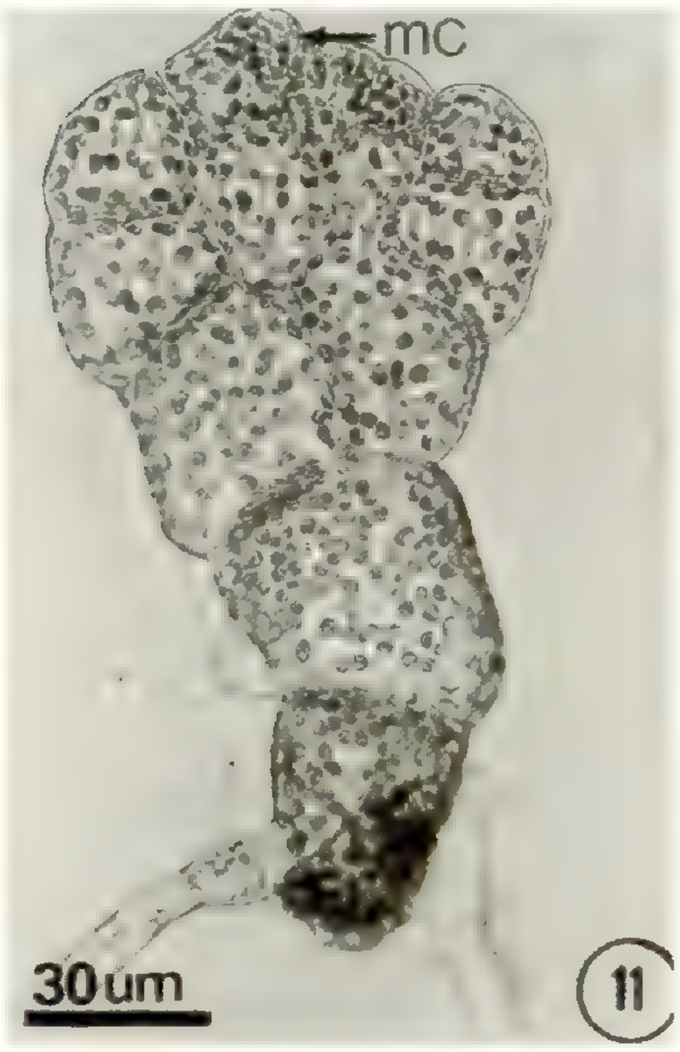
The filament phase begins between days 11 and 26. During this phase a sequential series of cell divisions occur in essentially the same plane to produce a slim, pluricellular germinal filament of three to seven barrel shaped cells (Figs. 5–9). All of these prothallial cells have numerous chloroplasts. This uniseriate condition is only a temporary phase. *Thelypteris oviedoae*, *T. piedrensis*, *T. reptans* var. *reptans* and *T. tetragona* have the same development period of 11–15 days; the development of this phase takes longer in *T. dissimulans* (11–26 days).

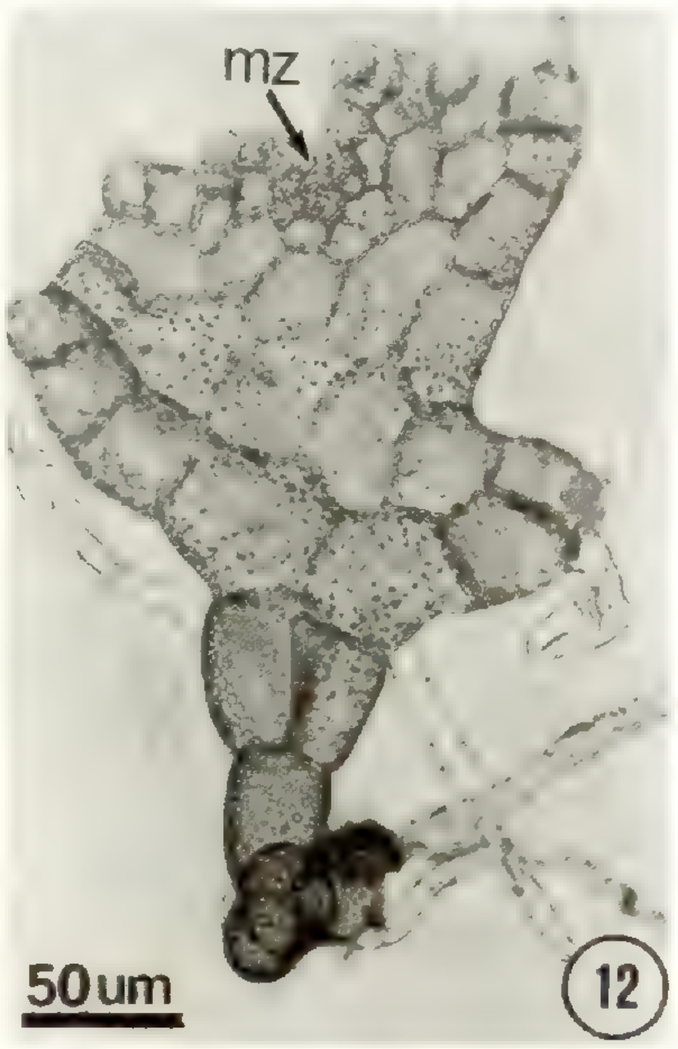
The development time of the plate phase varies among species: in *T. oviedoae* and *T. piedrensis* it is 26–50 days long, in *T. reptans* var. *reptans* and *T. tetragona* 26–60 days, and for *T. dissimulans* it is 70 days. All species have an *Aspidium*-type prothallial development (Fig. 10–13), in which the uniseriate filament and plate phase development begins before the appearance of hairs, the meristematic cell is located centrally (Fig. 11) along the distal margin, the prothallus grows due to this cell's activity and later acquires a notch and becomes apical, a pluricellular meristem becomes apparent and, later, a cushion develops; young prothallia begin to grow unicellular (Fig. 12), capitate hairs.

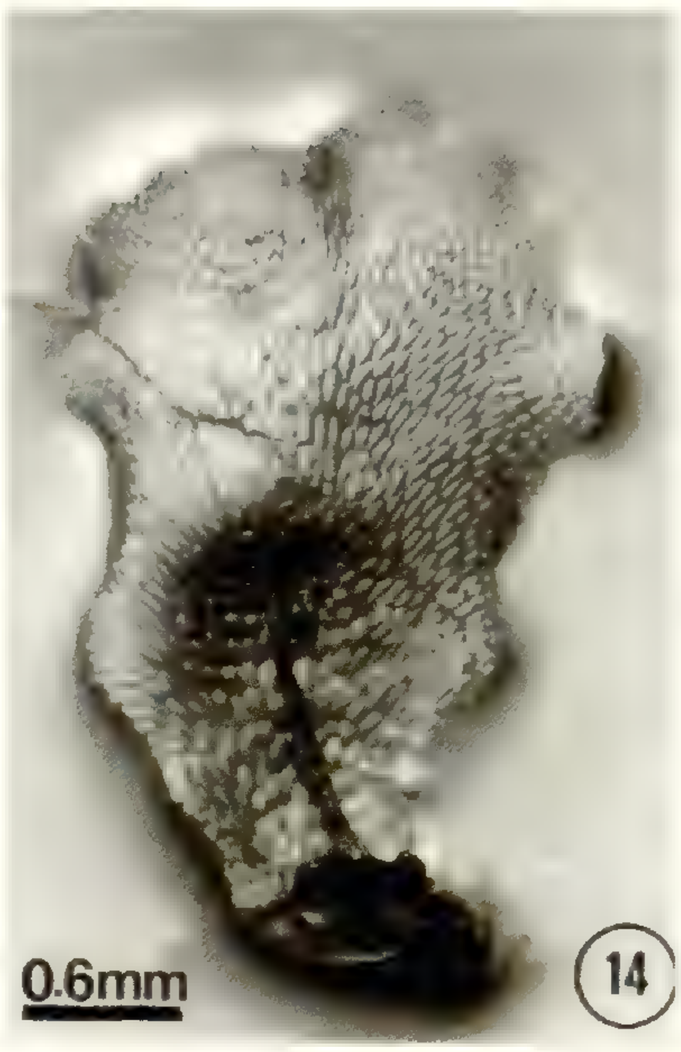
The *Aspidium*-type is variable with regard to sequence of cell divisions. The sequence of cell divisions and development of the young thallus are conditioned here by early hair formation in the young prothalli. Plate phase formation is initiated by cells behind the terminal cell (sometimes the penultimate cell of the germ filament in also sluggish) dividing longitudinally. Marginal hairs are produced continuously.

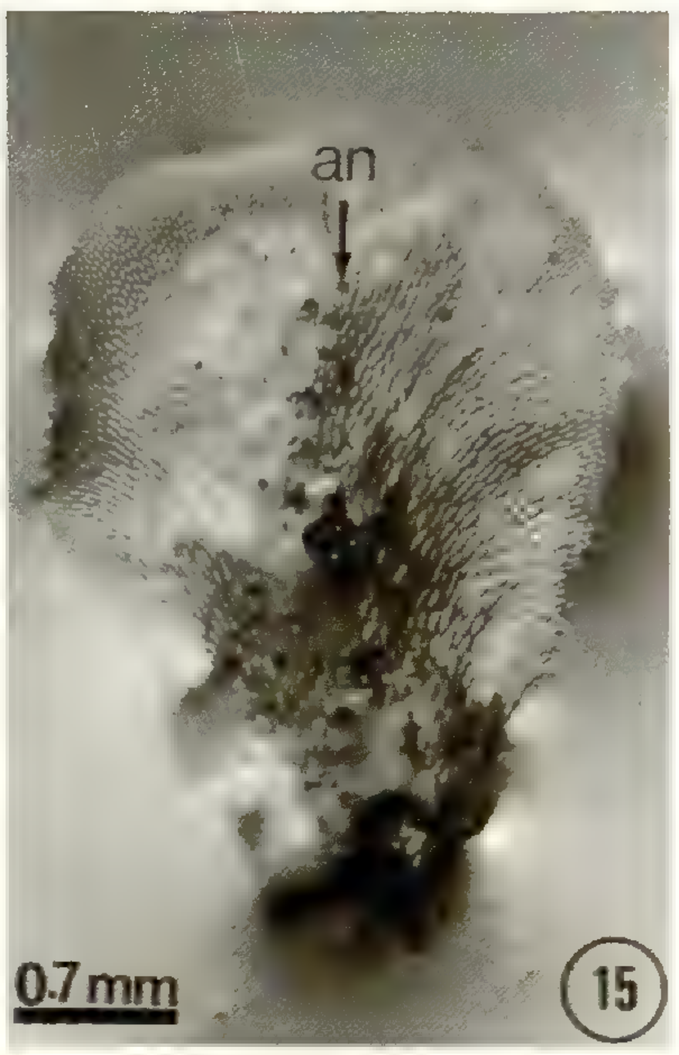
The prothallus grows by the activity of the meristematic cell and soon the meristematic region becomes notched and apical by unilateral growth of the thallus. A pluricellular meristem is established in the usual way and soon afterwards a midrib is formed. The mature prothallus is cordate and profusely hairy.

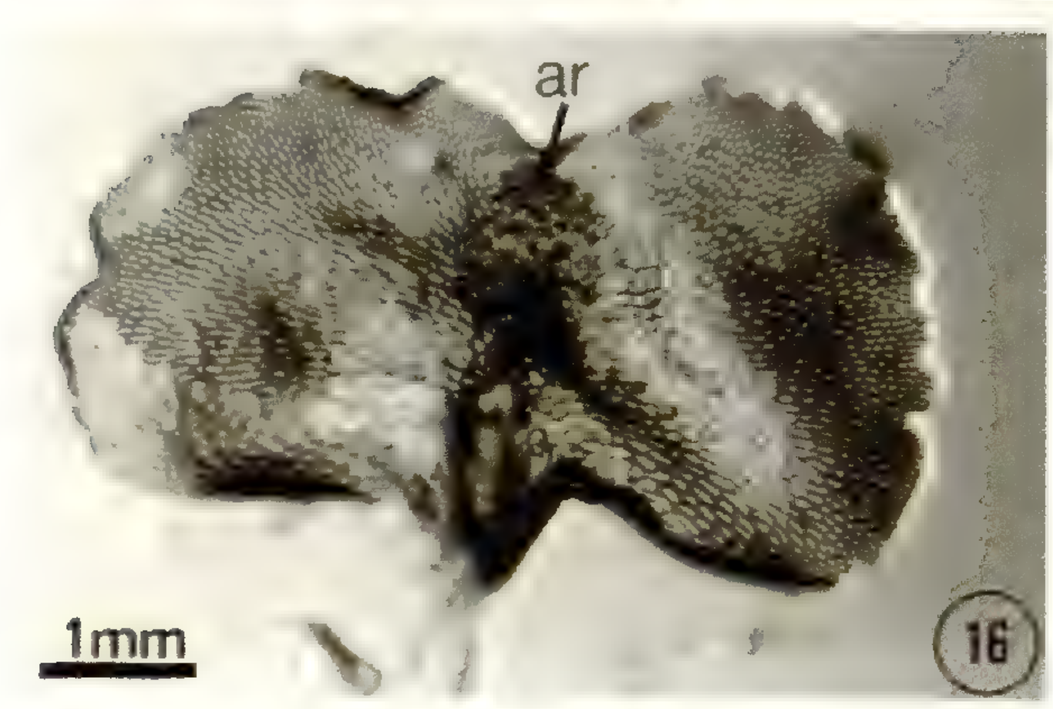
The adult phase corresponds to days 60 to 150 in *T. reptans* var. *reptans* and *T. tetragona*. It has a cordiform-spatulate shape with an indistinct notch, an elongated, thickened cushion over which the gametangia and rhizoids are found, and short, more or less isodiametric, undulating wings with marginal and superficial capitate hairs (Figs. 14–15). There is variation in the shape of the gametophyte in *T. oviedoae* and *T.piedrensis* (50–75 days), *T. reptans* var. *reptans* (80–150 days), and *T. dissimulans* and *T. tetragona* (60–200 days) where the gametophytes have a cordiform-reniform shape, with a dense cushion, a marked notch, wide and isodiametric wings, with undulate margins and the same type of hairs (Figs. 16–17). Rhizoids of all gametophytes are ventral, unicellular, hyaline to light brown, and abundant, intermixing with the reproductive structures.

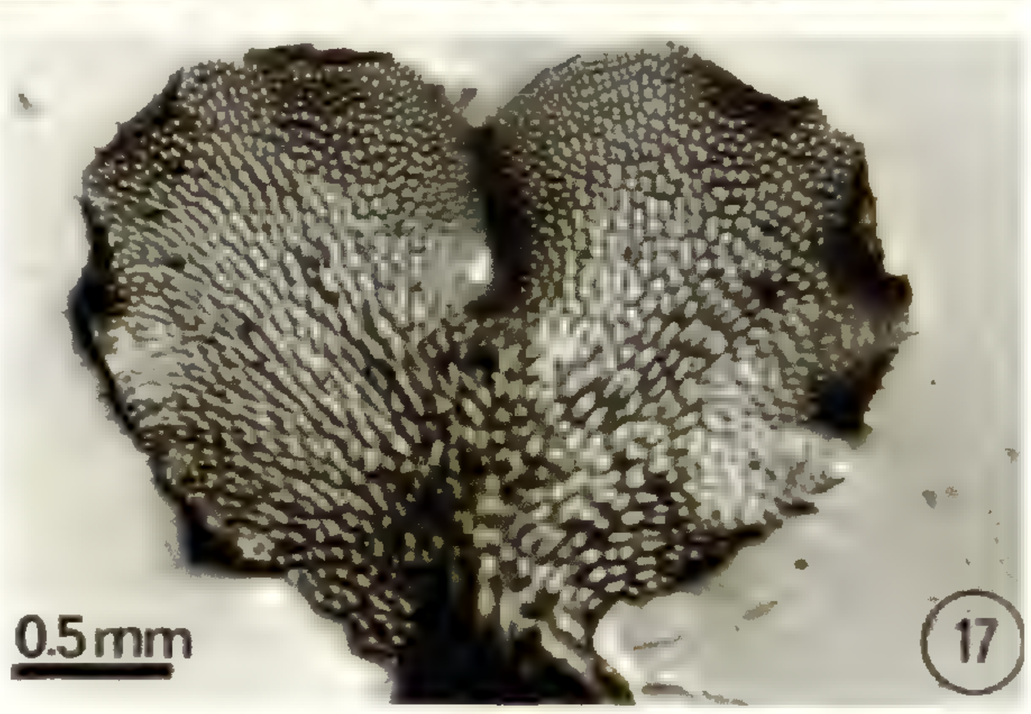












FIGS. 10–17. Developmental stages of the gametophytes of *Thelypteris*. 10–13. Young plate phases. 10. *T. piedrensis* (15 days). 11. *T. tetragona* (15 days). 12. *T. tetragona* (26 days). 13. *T. piedrensis* (32 days). 14–17. Adult gametophytes, spatulate to cordiform. 14. *T. tetragona* (89 days). 15. *T. tetragona* (70 days). 16. *T. reptans* (89 days). 17. *T. piedrensis* (50 days). an = antheridia, h = secretory hairs, mc = meristematic cell, mz = meristematic zone.

According to Atkinson, thelypteridoid ferns are generally characterized as having pubescent gametophytes; glabrous gametophytes have never been observed. Hairs are commonly simple and non-chlorophyllous, with a yellow or transparent secretion. Hairs of the studied species were found on the margin and on both surfaces of the gametophyte; they are hyaline unicellular, and capitate, with an extracellular waxy coat on the apex (Figs. 18–19). These hairs develop during the young plate phases in *T. oviedoae* and *T. piedrensis*, in *T. reptans* var. *reptans* at 26 days and in *T. dissimulans* and *T. tetragona* at 35 days. They are abundant on the adult phase, and are distributed along the margin and on both surfaces.

The gametangia are characteristic of typical leptosporangiate ferns. Archegonia are located distally on the cushion intermingled with the rhizoids. The long neck, which is oriented toward the meristematic zone, is composed of four rows of neck cells and a mouth of four cells (Figs. 20–21). Archegonia develop in *T. reptans* var. *reptans* at 67–75 days, in *T. piedrensis* at 89 days and in *T. tetragona* at 70–108 days. The antheridia are small, globose, with a basal cell, an annular cell and an opercular cell (Figs. 22–23). They develop in *T. tetragona* at 70–108 days, in *T. piedrensis* at 89 days and in *T. reptans* var. *reptans* at 67–108 days. Gametangia did not develop in either *T. disimulans* or *T. oviodeae* during the 300 days of cultivation.

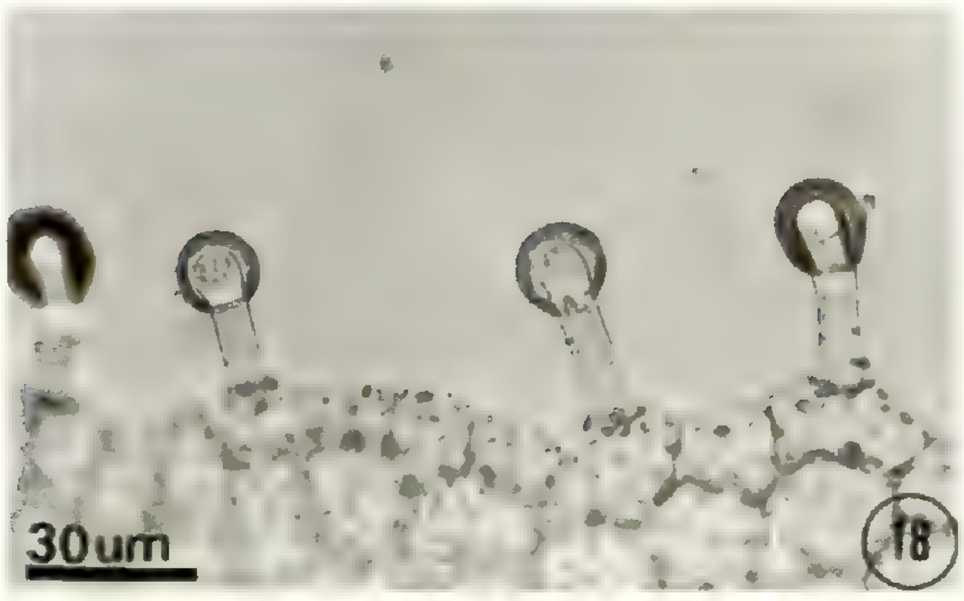
Sporophyte initiation was observed in *T. tetragona* at 89–166 days, and in *T. reptans* var. *reptans* at 137–285 days. Sporophytes did not develop in cultures of *T. dissimulans*, *T. piedrensis* or *T. oviedoae*, even after 300 days of cultivation.

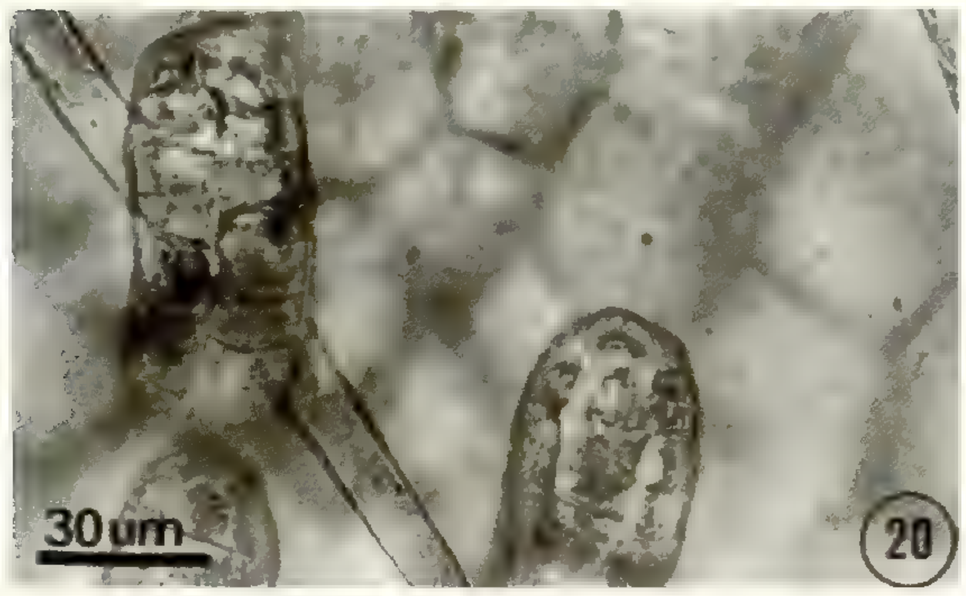
First leaves of young sporophytes had a petiole and a 2-, 3-, or 4- lobed blade. The venation was open and dichotomous (Figs. 24–26), All leaves had abundant hairs, marginally and on both surfaces. The hairs were unicellular, capitate, hyaline, achlorophyllous and secretory, similar to those on young and adult gametophytes. Stomata are of the policitic- type, with one or two subsidiary cells (Kondo, 1962; Thurston, 1969; Smith, 1990).

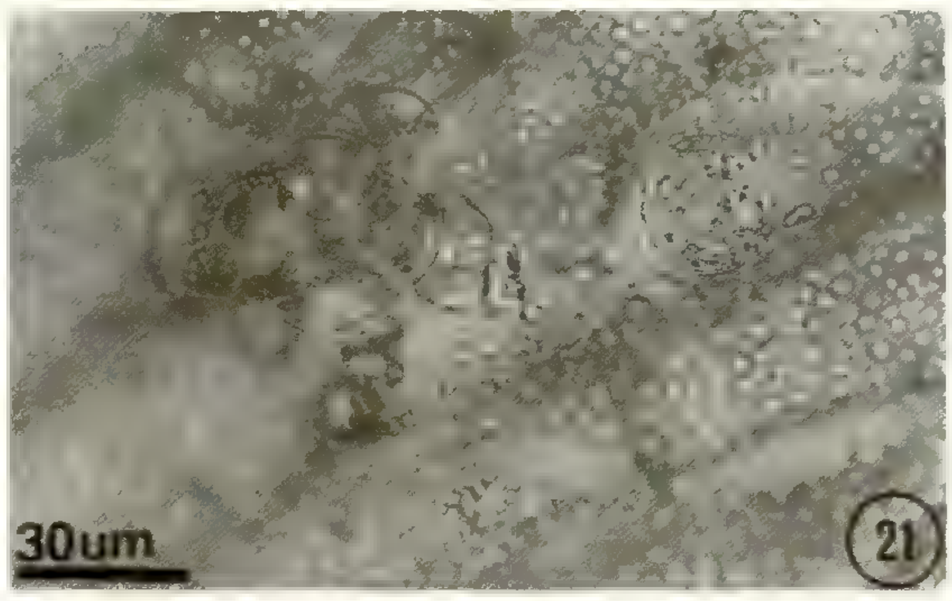
Discussion

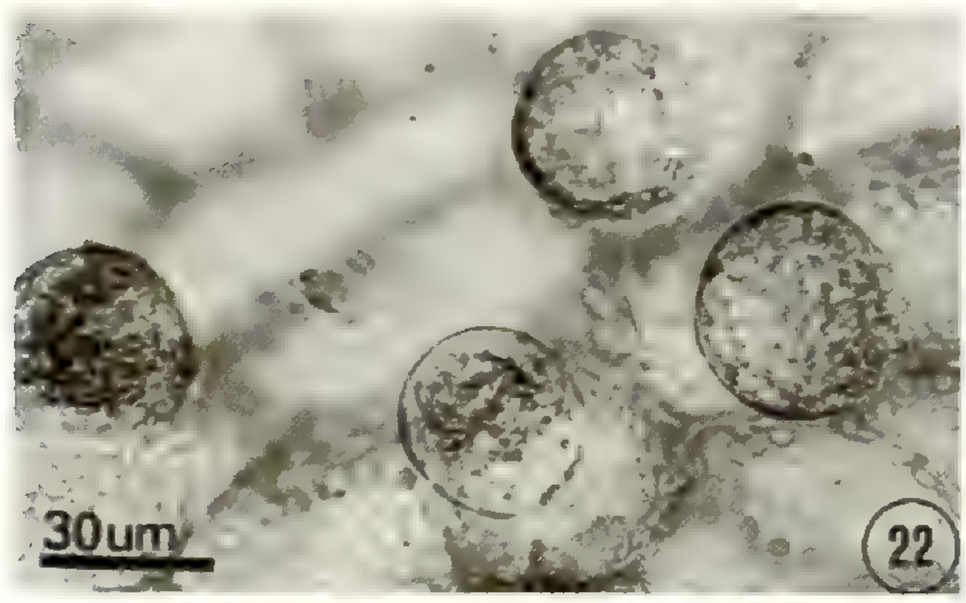
All spores are monolete, with ornamentation ranging from reticulate-pitted in *Thelypteris* subgenus *Amauropelta*, to winged in *T.* subgenus *Goniopteris*, to low ridged or ridged-papillate in *T.* subgenus *Cyclosorus* (Wood, 1973; Tryon and Tryon, 1982; Tryon and Lugardon, 1991).

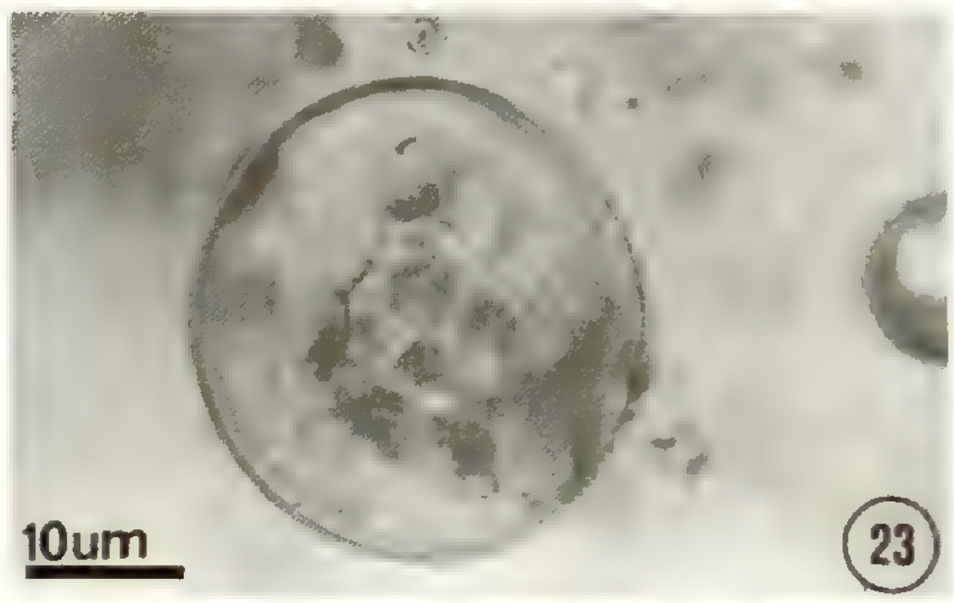
The gametophyte phase of the Thelypteridaceae, especially subgenera *Amauropelta*, *Cyclosorus*, *Goniopteris* and *Thelypteris*, is notoriously uniform, both in relation to spore germination, the *Vittaria*-type (Nayar and Kaur, 1971; Huckaby and Raghavan, 1981a) and prothallial plate development, the *Aspidium*-type. In the *Vittaria*-type germination, the spore's first division produces two unequal cells, a smaller one that immediately elongates and differentiates into the first rhizoidal cell, and a second larger cell which eventually gives rise to cells of the prothallial plate.

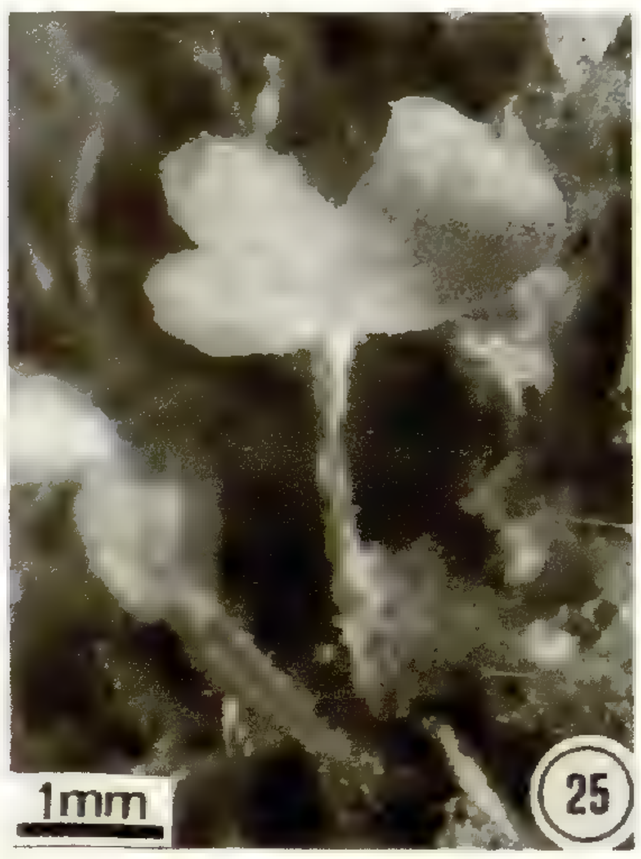












FIGS. 18–26. Secretory hairs, gametangia and first leaves in *Thelypteris*. 18–19. Unicellular secretory hairs. 18. *T. piedrensis* (89 days). 19. *T. reptans* (75 days). 20–21. Archegonia. 20. Neck of archegonia in *T. reptans* (67 days). 21. Mouth of archegonia in *T. tetragona* (70 days). 22–23. Antheridia. 22. *T. reptans* (67 days). 23. *T. tetragona* (108 days). 24–26. First sporophyte leaves. 24. *T. reptans* (137 days). 25. *T. tetragona* (166 days). 26. *T. reptans*. (166 days).

Seven different types of prothallial development in the homosporous ferns are described by Nayar and Kaur (1969). The critical elements which define these types are differences in the sequence of cell divisions during development, the location at which a meristematic cell or pluricellular meristem is established, and the ultimate shape of the thallus.

Although the taxa studied by us show a delay in prothallial hair development according to Nayar and Kaur (1969), we consider that they have an *Aspidium*-type development because the meristematic cell has a very active role in the growth and expansion of the young prothallus and this *Aspidium*-type of development is seen in more advanced genera such as *Thelypteris*.

During the each developmental stage, the plate of the gametophyte originates from three divisions on the same plane - two lateral and one horizontal - of the inferior face of an initial meristematic cell. That cell is subsequently replaced by a group of meristematic cells which contribute to the development of the cushion and to the expansion of the gametophyte wings. The substitution of the initial cell by a pluricellular meristem is common in other subgenera of *Thelypteris* (Kachroo, 1963; Nayar and Chandra, 1963; Atkinson, 1971; Reyes-Jaramillo and Pérez-García, 1991).

A distinctive characteristic of the gametophytes of the different subgenera of the Thelypteridaceae is the differentiation of unicellular, short, capitate hairs, with a wax secretion at the apex, during the blade development. This was clearly seen in all species.

Various authors have studied the gametophyte morphology of various species of Thelypteridaceae. They are all pubescent, with a relatively long-lived thallus that become cordate at maturity, delicate for their size, with a cushion and wide wings, abundant colorless or light brown rhizoids and advanced-type sexual organs. Although the differences regarding spore ornamentation, length of simple hairs and antheridia shape are insignificant, these characteristics vary in different species; the taxa we studied share the characteristics previously mentioned. We summarize that the morphology of the gametophytes in *Thelypteris* have a *Vittaria* type germination pattern, an *Aspidium* type prothallial development, that the gametophytes are generally uniform in form and development, and show enough diversity to suggest that they are useful in understanding taxonomic boundaries.

In the species studied here we only observed the most common type of hair, which is unicellular, capitate, secretory and with a waxy secretion; we did not observe other types of hairs mentioned in the literature, for example, secretory hairs, long, glandular-septate hairs, and acicular hairs (Kachroo, 1963; Nayar and Kaur, 1971; Tigerschiöld, 1989b, 1990).

Prothallial hairs have developed only in some subgenera, possibly the presence of similar types of hairs in groups apparently unrelated gave origin to the idea that epidermal derivates are of little value in taxonomic and phyletic studies. Nevertheless, the restricted distribution of pubescent gametophytes among several phyletic groups, and the presence of certain characteristic types of hairs in other groups (for example: acicular hairs in the Thelypteridaceae) apparently indicate that these are important characteristics in comparative

studies. As is common with other aspects of gametophyte morphology, the tendency to have hairs seems to have developed independently in the different phyletic groups of ferns.

Regarding the gametangia, our results indicate that the antheridia have the characteristic type of structure and ontogeny characteristic of advanced ferns such as the Thelypteridaceae (Davie 1951) and Stone (1960). Tigerschiöld's (1989a) contributions in which she mentioned four different dehiscence types in antheridia: 1) irregular split on the wall of the opercular cell, 2) a circular split similar to a pore, 3) the opercular cell is expulsed completely to the exterior and 4) the slit opens laterally (like a lid), the operculum remains joined to the annular cell. Although it has been stated that dehiscence is specific to each species, Tigerschiöld (1989a) observed that this is not always true, the same species can have two or three of these dehiscence mechanisms. All of the gametophyes in this study dehisced by means of the separation of the whole opercular cell, which in this case corresponds to Tigerschiöld's type three. In other studies, Tigerschiöld (1989b, 1990) showed that his thelypteroid ceylandes gametophytes are similar in most respects.

Nevertheless, we did find some distinguishing characteristics among the species. Variation was present in thallus margin shape, hair length, abundance of secretory hairs, presence or absence of acicular hairs, number of archegonial neck cells, and opercular cell form. Just as antheridial anthesis and antherozoid liberation varies in each of the species studied, this study demonstrates that it is possible to identify species based on characteristics of the gametophyte alone.

Stokey (1951), Atkinson and Stokey (1964) and Smith (1971) state that fern gametophytes have only recently begun to be considered as a possible source of taxonomic characters, or as a comparative morphological tool. Gametophytes seem to be useful in systematics at the family level, and in certain cases, at the generic level, but they are most frequently used, on a smaller scale, to distinguish species within the same genus.

The gametophyte's uniform development in species of different subgenera of the Thelypteridaceae has been mentioned by several authors (Nayar and Chandra, 1963; Huckaby and Raghavan, 1981a; Tigerschiöld, 1989a, 1989b, 1990; Reyes-Jaramillo and Pérez-García, 1991). Nevertheless, the comparative study of the gametophyte development of American species helps define, based on hair type and position, margin, antheridial structure and shapes of the anteridial slit, the combination of characters that will delimitate subgenera, species, or groups of species within the Thelypteridaceae (Atkinson 1973; Pryer *et al.*, 1995).

This study of the gametophytes of five species of thelypteroid ferns from the New World indicates that they can be very diverse and that they are distinctive. Nevertheless, because the sampling was scarce, this conclusion could be premature or it could only represent a record of the gametophyte's characteristics and an effort to accommodate or adjust them within today's classification. This research could be useful as a comparative basis in which many thelypteroid gametophytes could begin to be known.

ACKNOWLEDGEMENTS

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Shorter Notes

***Botrychium pallidum* Newly Discovered in Maine.**—A recent report of *Botrychium pallidum* W. H. Wagner from Maine (Wagner, Jr., W. H. and F. S. Wagner, 1993, Ophioglossaceae. Pp. 85-105 in FNA Editorial Committee, *Flora of North America north of Mexico, vol. 2. Pteridophytes and Gymnosperms*. Oxford University Press, New York) was apparently erroneous (W. H. Wagner, pers. comm.) and was subsequently removed in the second printing of early 1994. It is not known on what basis the report was made, and no specimens have been seen in regional herbaria to support it. Now, however, the species has been discovered at a site in Washington County, Maine, the first in the northeastern United States. The site is located approximately 440 km south of the nearest known station at Bic, Saguenay County, Quebec.

The Maine population was observed first on 3 July 2000 and again on 17 June 2001. On both dates, only two plants were observed. Given the difficulties inherent in identifying moonworts (Williston, P. 2001. *The Botrychiaceae of Alberta*. Alberta Natural Heritage Information Centre, Edmonton), the identification awaited confirmation until another visit was made on 14 June 2003, when 60 well-formed, unequivocally identifiable plants were observed. They occurred singly and in small clumps (2–4 stems per clump), scattered over an area approximately 12 m × 60 m. On a return visit on 24 June 2003, fewer plants were observed, perhaps due to herbivory; plants on that date were observed to be sporulating and most were already senescent. One plant (above ground parts only) was collected for isozyme analysis and two for vouchers. Isozyme analysis also confirmed the identity (Don Farrar, Iowa State University, pers. comm.).

Voucher material: **USA**: Maine: Washington County: Steuben, Petit Manan National Wildlife Refuge, 60 plants in old field, with *Botrychium simplex* and *Botrychium matricariifolium*, among low grasses, forbs, mosses, and lichens, 14 June 2003, *Gilman 03008, Norm Famous & Marcia Spencer-Famous* (IA); 24 June 2003, *Gilman 03009, Arthur Haines, Sally Rooney, Linda Welch & Michael Langlois* (MAINE, Herb. Petit Manan National Wildlife Refuge).

Botrychium pallidum was originally described from Quebec, where it was collected near Baie St. Catherine (Wagner, Jr., W. H. and F. S. Wagner. 1990. Notes on the fan-leaved group of moonworts in North America with descriptions of two new members. Amer. Fern J. 80:73–81). In Quebec, it has also been collected at Bic (*Wagner 90010 & Wagner*, MICH, image seen; *Coursol et al.*, DAO, image seen), and Forillon Federal Park, Gaspé County (J. Labrecque, Ministère de l'Environement du Quebec, pers. comm.). It is also known from the Great Lakes region in Michigan (Upper and Lower Peninsulas), Ontario, and Minnesota, and occurs in Saskatchewan, Alberta, Montana, and Colorado (Wagner and Wagner, 1990; Wagner and Wagner, 1993; Williston, 2001). Nevertheless, it is a rare species with widely scattered populations normally

consisting of few individuals. It currently has a Global Heritage Status Rank of G3 (globally vulnerable) taxon (NatureServe, 2003, NatureServe Explorer: an online encyclopedia of life [web application]. Version 1.8. NatureServe, Arlington, VA. http: www.natureserve.org/explorer accessed 6 April 2004). Williston (2001), however, suggested it may be even rarer, i.e., G2 (globally imperiled).

The Maine population occurs on the Petit Manan National Wildlife Refuge (PMNWR), a federal holding dedicated primarily to preserving migratory bird habitat and enhancing the success of seabird nesting (http: petitmananfws.gov). The *Botrychium pallidum* colony is approximately 400 m from the ocean, in a dry field with a sparse to thin stand of grasses, notably *Festuca filiformis* Pourret and *F. rubra* L., and a sparse cover of mostly non-native forbs, especially *Hieracium pilosella* L., *H. praealtum* Gochnat, *Taraxacum laevigatum* (Wild.) DC., *Rhinanthus crista-galli* L., *Rumex acetosella* L., and an undetermined annual species of *Trifolium*. Co-occurring in the habitat are *Botrychium matricariifolium* A. Br. and *B. simplex* E. Hitchc.

Other fields on the Refuge show a significant or dominant cover of ericaceous shrubs, e.g. *Vaccinium angustifolium* Aiton, *V. vitis-idaea* L., *V. myrtilloides* Michx., *Kalmia angustifolia* L., and *Rhododendron canadense* (L.) Torr. However, only a few scattered plants of *Vaccinium angustifolium* and *V. vitis-idaea* occur in the area of the *Botrychium* colony, evidence perhaps of a less acidic soil reaction or different history of disturbance in the immediate area. Areas within the colony not covered with vascular plants support patches of mosses, including *Aulacomnium palustre* (Hedw.) Schaegr. and *Polytrichum piliferum* Hedw., and lichens, *Peltigera* sp. and *Cladonia* spp.

The discovery presents challenges. One is to discover other regional populations. *Botrychium matricariifolium*, *B. simplex*, and *B. lunaria* (L.) Sw. all occur along the immediate coast and on islands in eastern Maine and the Maritime Provinces, and additional populations of *B. pallidum* are to be expected. Accurately characterizing the micro-habitat of this population with respect to soil type, soil chemistry, nutrient input (perhaps including ions from storm spray), disturbance regime, and other factors may prove helpful in searching for other regional populations.

Another challenge devolves upon the US Fish and Wildlife Service: to manage the habitat in a manner that will maintain this population. Some type of long-rotation disturbance regime may be necessary to retard habitat succession and provide colonizable sites (Gilman, A. V. 2002. *Botrychium lunaria (L.) Sw. (Moonwort) Conservation and Research Plan for New England*. New England Wild Flower Society, Framingham, MA).

Thanks are extended to Linda Welch, Refuge Manager, for permission to enter protected areas and to collect specimens, to Michael Langlois (PMNWR) for aid and information, to Norm Famous and Marcia Spencer-famous for assistance in fieldwork, and to Don Farrar, Iowa State University, for confirmation of a specimen through isozyme analysis. Janis Lesbines, Arthur Haines, and Sally Rooney also accompanied me in the field at various times.—ARTHUR V. GILMAN, William D. Countryman Environmental Assessment & Planning, 868 Winch Hill Road, Northfield, VT 095663.

***Asplenium ruta-muraria* L. in Iowa.**—A small population of *Asplenium ruta-muraria* L., a species previously unrecorded from Iowa (Eilers, L. J. & D. M. Roosa. 1994. *The Vascular Plants of Iowa*. University of Iowa Press, Iowa City; Peck, J. H. 1982. Ferns and fern allies of the Driftless Area of Illinois, Iowa, Minnesota and Wisconsin. Milwaukee Public Mus. Contr. Biol. Geol. 53:1–140; Peck, J. H. 1989. Additions to the Iowa Pteridophyte Flora, III. J. Iowa Acad. Sci. 96:54–56), was recently discovered by the first author in Clinton County in the east-central part of the state. This locality is some 300 miles north of its nearest known location in the Missouri Ozarks (Wagner, Jr., W. H., R. C. Moran and C. R. Werth. 1993. Aspleniaceae Newman. Pp 228–245 in FNA Editorial Committee. *Flora of North America North of Mexico. Vol. 2 Pteridophytes and Gymnosperms*. Oxford Univ. Press, New York; Yatskievych, G. 1999. *Steyermark's Flora of Missouri, Vol 1. Revised edition*. Missouri Botanical Garden Press, St. Louis.). Since the Iowa population is small (> 50 plants), only portions of a few plants were collected and these were deposited in the University of Iowa Herbarium (Clinton Co., Iowa: *T. F. Cady s.n.*, May 18, 2001, IA).

Primarily an Appalachian species in North America (Fig. 1), the main range of *A. ruta-muraria* is from southeastern Ontario, south-central Quebec, New York, Vermont, New Hampshire and Massachusetts southwest into Kentucky, Tennessee, Georgia and Alabama (Wagner, *et al.*, 1993; Monro, D. 1988. A disjunct station of *Asplenium ruta-muraria*, with *Pellaea atropurpurea* and *P. glabella*, in Eastern Ontario. Amer. Fern J. 78:136–138). Additionally, there are a number of disjunct populations. The most extensive of these are in the Ozarks in southeastern Missouri (Yatskievych, 1999) and along the Niagara Escarpment in Lake Huron. In the nineteenth century, the Ozark population was reported to extend into adjacent Arkansas, but no specimens or extant populations are known (Taylor, W. C. 1984. *Arkansas Ferns and Fern Allies.* Milwaukee Public Museum, Milwaukee). The Ozark population also may have reached into Illinois. Mohlenbrock (Mohlenbrock, R. H. 1999. *The Illustrated Flora of Illinois. Ferns. Second Edition*. Southern Illinois University Press, Carbondale) reported that *A. ruta-muraria* was collected from an unspecified location in southern Illinois in the mid 1800's, but it is now presumed extirpated from the state. The Niagara Escarpment populations in southwestern Ontario extend through the Bruce Peninsula and Manitoulin Island to Drummond Island in Michigan's Upper Peninsula (Monro, 1988; Drife, D. C. & J. E. Drife. 1990. Oliver A. Farwell's early Pteridophyte records from the Keweenaw Peninsula. Michigan. Bot. 29:90–91; Gart Bishop pers. com.). An additional Michigan population previously reported from Keweenaw County (Monro, 1988; Wagner *et al.*, 1993) is discounted (Drife & Drife, 1990). Another remarkable disjunction was recorded recently by Gart Bishop and Bruce Bagnell from New Brunswick at Kennebecasis Bay, Kings County (Sheppard, M. 2001. In awe of minister's face, trust buys rare plant habitat. Refuge 11(2):1; Gart Bishop, pers. com.). The Iowa population augments the picture of a species characterized by widespread disjunctions well outside the core Appalachian range.

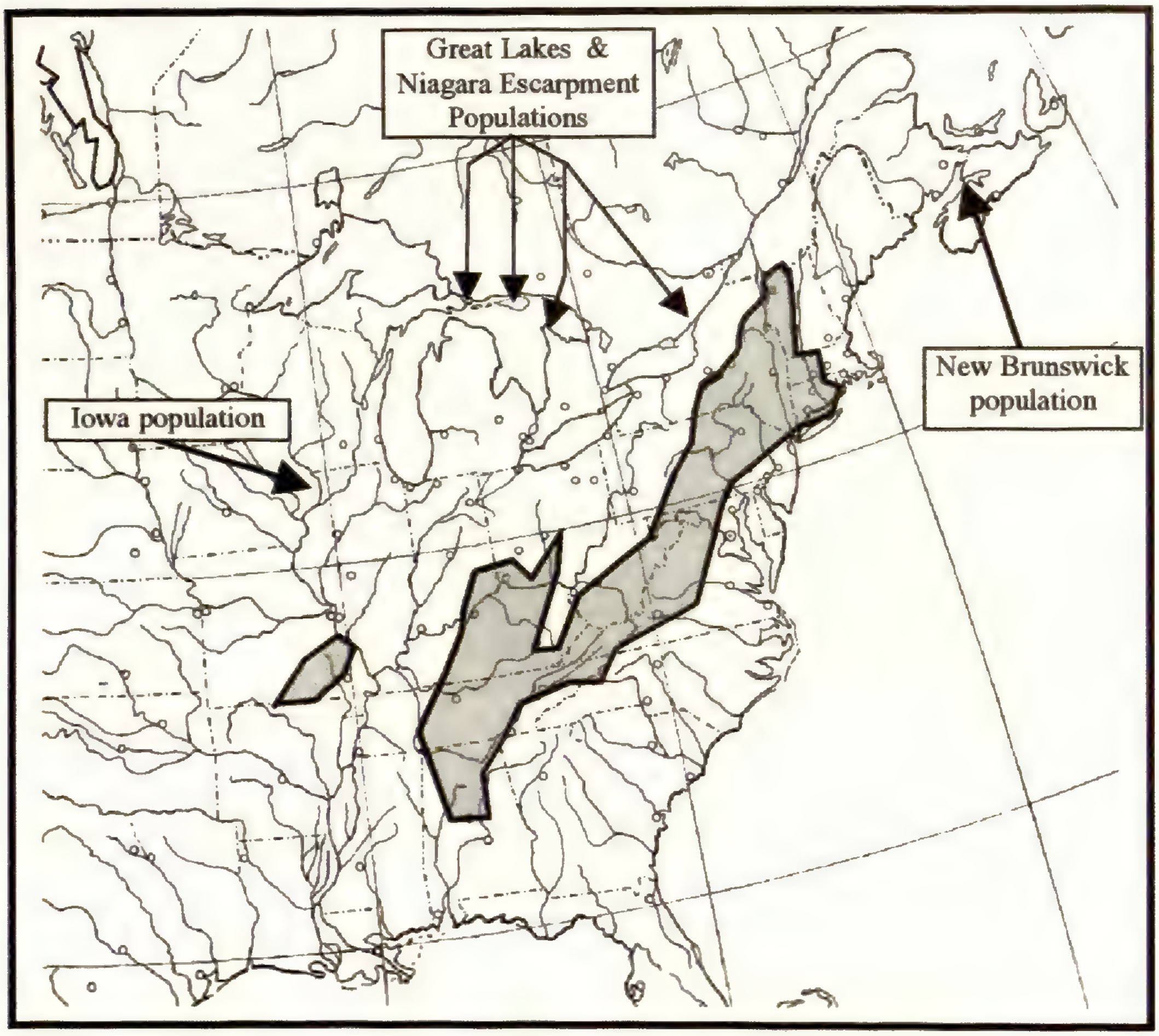

FIG. 1. North American distribution of *Asplenium ruta-muraria*, including the newly discovered Iowa population. Map based on sources cited in text.

The Iowa population of *A. ruta-muraria* occurs along the edge of a dolomite prairie characterized by *Schizachyrium scoparium* (Michx.) Nash (little bluestem), *Bouteloua curtipendula* (Michx.) Torrey (sideoats grama), *Sporobolus heterolepis* (Gray) Gray (prairie dropseed), *Carex richardsonii* R. Br., *C. umbellata* Schkuhr *ex* Willd., *Liatris cylindrica* Michx. (blazing star), *Arabis lyrata* L. (rockcress), *Dalea purpurea* Vent. (prairie clover), *Aster sericeus* Vent and *A. azureus* Lindley, to name but a few. The southern and western perimeters of this prairie are defined by dolomitic outcrops; *A. ruta-muraria* occurs near the southeastern edge on small cliffs approximately 2.5–3.5 meters high. Although there has been some minor woody encroachment in recent history, these bluffs were historically surrounded by prairie, based on descriptions in the General Land Office Survey of 1837 (General Land Office Survey Field Notes, Iowa City, IA, State Historical Society of Iowa). The main population occurs on a section line, so the surveyor, John Wharry, would have walked right through this site and his notes recorded no trees or shrubs, only

"Broken along the line 2nd rate Land generally, Lime stone rock along the ravines".

The largest concentration of *A. ruta-muraria* at this site occurs in a small, exposed area at the eastern terminus of a south-facing outcrop. On the south and east facing bluffs, *A. ruta-muraria* grows with the dominant fern encountered on these outcrops, *Pellaea glabella* Mett. *ex* Kuhn (smooth cliff-brake), whereas on the small, sheltered northern face it occurs with *Cystopteris bulbifera* (L.) Bernh. (bulblet fern). Other plant species co-inhabiting the shelves and crevices of these bluffs include *Arabis lyrata* (rock cress), *Euphorbia maculata* L. (spurge), *Minuartia michauxii* (Fern.) Farw. (=*Arenaria stricta* Michx.; rock sandwort), *Nepeta cataria* L. (catnip), *Parietaria pensylvanica* Muhl. *ex* Willd. (pellitory), *Aquilegia canadensis* L. (columbine), *Sporobolus neglectus* Nash (small rush grass) and *Schizachyrium scoparium* (little bluestem).

We thank Gart Bishop, Doug Larson, Jim Peck, Tony Reznicek, Carl Taylor, and George Yatskievych for information or advice.—THOMAS F. CADY, Iowa City, IA and DIANA HORTON, Biological Sciences Department, 143 BB, University of Iowa, Iowa City, IA 52242.

Vitexin 7-*O*-rhamnoside, a New Flavonoid from *Pteris vittata*.—Previous work on the flavonoids of *Pteris vittata* L. has led to the identification of luteolinidin 5–*O*-glucoside by Harborne (Phytochemistry 5:589–600, 1966); in addition acid hydrolysis of extracts of this fern has led to the identification of kaempferol, quercetin, leucocyanidin and leucodelphinidin by Voirin (Ph. D. thesis, University of Lyon, p. 151, 1970). More recently 3-*C*-(6‴-acetyl-β–cellobiosyl)-apigenin (Amer. Fern J. 89:217–220, 1999) and 6-*C*-β–cellobiosyl-isoscutellarein-8-methyl ether together with quercetin 3-*O*-glucuronide and rutin (Amer. Fern J. 90:42–45, 2000) have been identified by Imperato and Telesca. In addition three kaempferol glycosides (3-*O*-glucoside, 3-*O*-glucuronide and 3-*O*-(X″,X″-di-protocatechuoyl)-glucuronide), two di-*C*-glycosylflavones (3,8-di-*C*-arabinosylluteolin and 6-*C*-arabinosyl-8-*C*-glucosylluteolin) and three flavonol glucosides acylated with hydroxycinnamic acids (kaempferol and quercetin 3-*O*-(2″, 3″-di-*O*-*p*-coumaroyl)-glucosides together with kaempferol 3-*O*-(X″-*O*-*p*-coumaroyl-X″-*O*-feruloyl)-glucoside) have been found by Imperato (Amer. Fern J. 90:141–144, 2000; Amer. Fern J. 92:244–246, 2002; Amer. Fern J. 93:157–160, 2004).

In the present paper two flavonoids (I and II) have been isolated from aerial parts of *Pteris vittata* L. collected in the Botanic Garden of the University of Naples. The fern was identified by Dr. R. Nazzaro (University of Naples); a voucher specimen (149.001.001.01) has been deposited in the Herbarium Neapolitanum (NAP) of the University of Naples.

Flavonoids (I and II) were isolated from an ethanolic extract of aerial parts of *Pteris vittata* by preparative paper chromatography in BAW (*n*-butanol-acetic acid-water, 4:1:5, upper phase), 15% HOAc (acetic acid) and BEW (*n*-butanol-

TABLE 1. ^{13}C- and ^{1}H-NMR spectral data (DMSO-d_6) of flavonoid (I).

Carbon	δ_c ppm	δ_H ppm (J in Hz)
Apigenin		
2	164.6	0.97 (3H, d, J = 6, rhamnosyl methyl group)
3	102.7	2.95–4.11 (10 H, m, glucosyl 6 protons + rhamnosyl 4 protons)
4	182.4	4.52 (1 H, d, J = 8, glucosyl H-1)
5	161.6	5.51 (1 H, d, J = 2, rhamnosyl H-1)
6	99.1	6.33 (1 H, s, H-6)
7	163.2	6.77 (1 H, s, H-3)
8	104.4	6.91 (2 H, d, J = 8..6,(??) H-3′ and H-5′)
9	156.8	7.97 (2 H, d, J = 8.6, H-2′ and H-6′)
10	103.2	
1′	121.6	
2′,6′	128.9	
3′,5′	115.7	
4′	160.5	
O-Rhamnosyl		
1″	99.2	
2″	70.4[a]	
3″	70.5[a]	
4″	71.8[b]	
5″	70.8[b]	
6″	18.2	
C-Glucosyl		
1‴	73.6	
2‴	71.5[b]	
3‴	79.6	
4‴	69.9	
5‴	82.1	
6‴	62.2	

[a,b] Assignments with the same superscripts may be interchanged.

ethanol-water, 4:1:2.2). Further purification was carried out by Sephadex LH-20 column chromatography eluting with methanol. Color reactions (brown to yellow in UV+NH_3.), chromatographic behaviour (R_f values on Whatman No 1 paper: O.37 in BAW; 0.54 in 15% HOAc) and ultraviolet spectral analysis in the presence of usual shift reagents (λ_{max}(nm) (MeOH) 273, 332; +$AlCl_3$ 280, 302, 344, 383; +$AlCl_3$/HCl 281, 300, 341, 381; +NaOAc 273, 391; +NaOMe 274, 391) suggested that flavonoid (I) may be a flavonoid glycoside with free hydroxyl groups at positions 5 and 4′. Acid hydrolysis (2N HCl; 1 hr at 100°C) gave L-rhamnose, vitexin (8-*C*-glucosylapigenin) and isovitexin (6-*C*-glucosylapigenin). These results show that flavonoid (I) may be a *C*-glucosylapigenin 7-*O*-rhamnoside and this was confirmed by the FAB mass spectrum which showed a quasimolecular ion at m/z 579 $[M+H]^+$ and an ion at m/z 601 $[(M+H)+Na]^+$. ^{1}H- and ^{13}C- NMR spectral data (Table 1) showed that D-glucose is attached at position 8 o f the flavone since H-6 appeared as a singlet at δ 6.33; in addition C-8 showed a downfield shift of 10.4 p.p.m., in comparison with the corresponding carbon atom of apigenin; this shift of glycosylated carbon

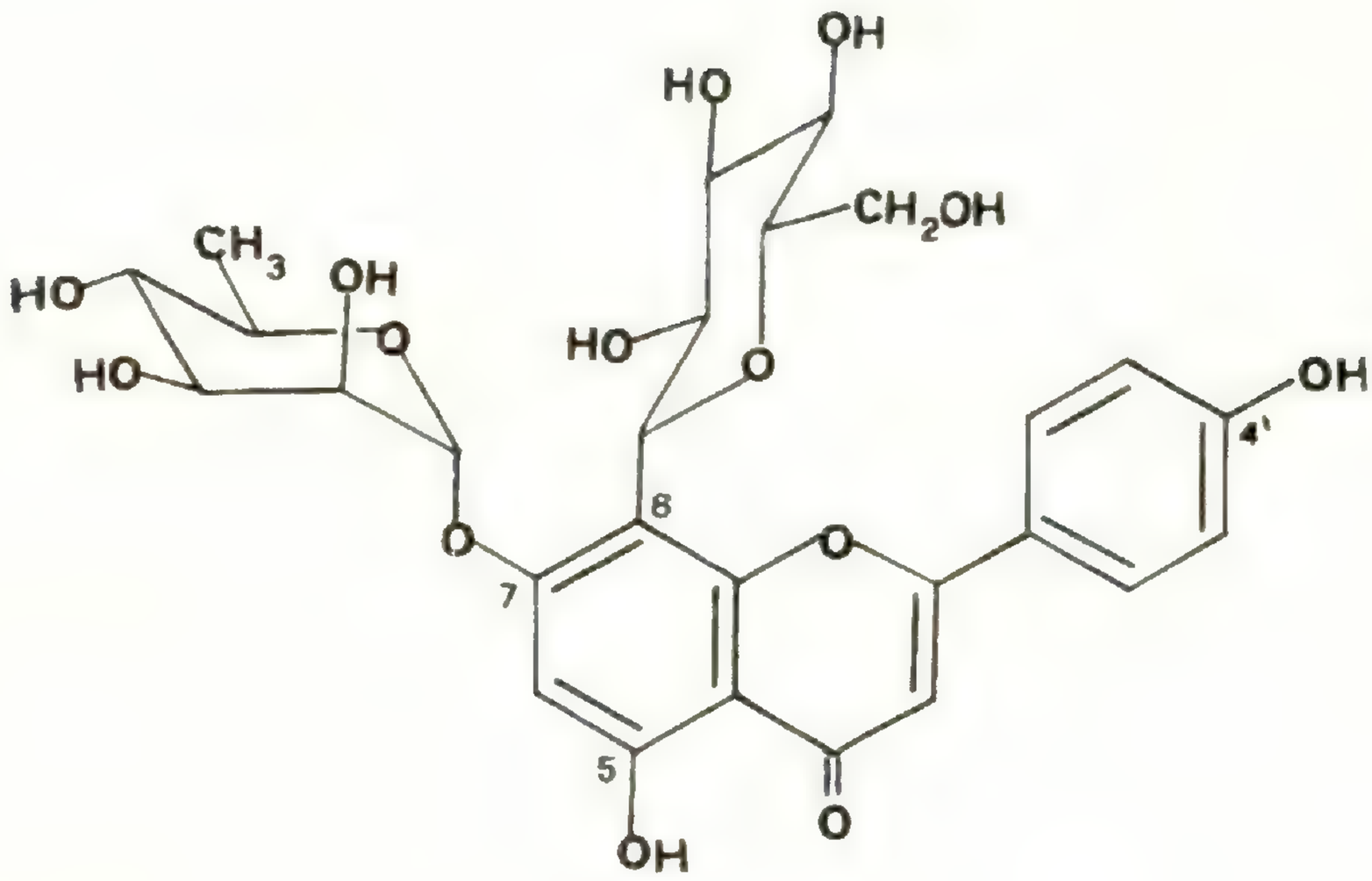

FIG. 1. Vitexin 7-*O*-rhamnoside.

atom is an effect of *C*-glycosylation on the aglycone spectrum as shown in the review by Markham and Chari (pp. 19–134 *in* J. B. Harborne and T. J. Mabry, eds., *The Flavonoids: Advances in Research*, Chapman and Hall, London, 1982). Hence the presence of isovitexin among the products of acid hydrolysis of flavonoid (I) is due to a Wessely-Moser acid isomerization. The combined data show that flavonoid (I) is vitexin 7-*O*-rhamnoside (Fig 1), a new natural product; ^{1}H- and ^{13}C-NMR spectral data (Table 1) support this structure.

A large number of *C*-glycosylflavonoids have been found in ferns as shown in the review by Markham (pp. 427–468 *in* J. B. Harborne, ed., *The Flavonoids, Advances in Research Since 1980*, Chapman and Hall, London, 1988) and in a review by Imperato (pp. 39–75 *in Current Topics in Phytochemistry*, Research Trends, Trivandrum, 2000). However *C*-glycosylflavonoid *O*-glycosides are rare in ferns. A C-glycosylflavonoid *O*-glycoside in which the hydrolyzable sugar is attached to a phenolic hydroxy group was found for the first time in ferns by Hiraoka (Bioch. Syst. and Ecol. 6:171–175, 1978) who identified vitexin 7-*O*-glucoside in the genus *Dryopteris;* subsequently 8-*C*-rhamnosylluteolin 7-*O*-rhamnoside was found in *Pteris cretica* (Phytochemistry 37:589–590, 1994) by Imperato. *C*-Glycosylflavonoid-*O*-glycosides in which the hydrolysable sugar is attached to a hydroxy group of a *C*-glycosyl moiety were found for the first time in ferns by Markham and Wallace (Phytochemistry 19:415–420, 1980) who found apigenin and luteolin 8-*C*-glucoside 2″-*O*-xylosides in *Trichomanes venosum*; two further *C*-glycosylflavone *O*-glycosides of this type have subsequently been found in *Pteris vittata* by Imperato and Telesca who identified these flavonoids as 3-*C*-(6‴-acetyl-cellobiosyl)-apigenin (Amer. Fern J. 89:217–220, 1999) and 6-*C*-β–cellobiosyl-isoscutellarein 8-methyl ether (Amer. Fern J. 90:42–45, 2000).

Flavonoid (II) has been identified as kaempferol 3-*O*-rutinoside by UV spectral analysis with the customary shift reagents, acid hydrolysis, ^{1}H-NMR

spectrum, ^{13}C-NMR spectrum and co-chromatography with an authentic sample. As shown in the review by Markham (1988), kaempferol 3-*O*-rutinoside has previously been identified in *Adiantum capillus-veneris* (Adiantaceae), another of the 53 species of *Adiantum, Loxsoma cunninghamii, L. costaricensis* (Loxsomaceae), all four species of *Bommeria* (Sinopteridaceae), four species of *Gymnopteris* (Sinopteridaceae), two species of *Hemionitis* (Sinopteridaceae) and the genus *Trachypteris* (Sinopteridaceae); more recently kaempferol 3-*O*-rutinoside has been identified in *Diplazium nipponicum* (Athyriaceae) and *Thelypteris palustris* (Sinopteridaceae) as shown in the review by Imperato (2000).

The author thanks Murst (Rome) for financial support. Mass spectral data were provided by SESMA (CNR, Naples).—Filippo Imperato Dipartimento di Chimica, Università della Basilicata, 85100 Potenza, Italy.

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A Taxonomic Study of the Fern Genus *Arachniodes* Blume (Dryopteridaceae) from China

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ABSTRACT.—The taxonomy of the fern genus *Arachniodes* Blume in China is rather complicated with the creation of many new names since the 1960's. The purpose of this article is to make a clarification of the genus as a whole from China and provide an enumeration of what is known at present. Through herbarium studies and field observations, the distribution, morphological criteria and subdivision of the genus *Arachniodes* from China are discussed. The total number of species has been reduced from ca 130 names to 58 names, some of which are still in need of studies to prove their acceptance. A subdivision of four sections is adopted and further completed, *i.e.* sect. *Cavaleria*, sect. *Globisorae*, sect. *Amoenae* , and sect. *Arachniodes*; and for the 50 species names included in the section *Arachniodes*, 10 species groups were proposed for the purpose of further comparison. Names of taxa that belong to *Arachniodes* known from China are enumerated in alphabetical order with information about their synonyms, their distribution and the sections and groups in which they are categorized.

The definition of the fern genus *Arachniodes* Blume is rather confused in that its species share some key characteristics with both *Dryopteris* Adans. and *Polystichum* Roth, the two largest genera of the family Dryopteridaceae. *Arachniodes* was established by Blume in 1828; however, the genus was not recognized by other pteridologists for nearly one and a half-centuries. Some of its species have experienced a lot of changes in nomenclature before Tindale (1961, 1965) resurrected *Arachniodes* as the acceptable generic name (Ching, 1934, 1938, 1962; Holltum, 1954; Morton, 1960; Ohwi, 1962). The subsequent circumscription and delimitation of the genus by Serizawa (1976), Proctor (1985, 1989), Wu and Ching (1991), and Hsieh (2000) is still incomplete; Sledge (1973) has called into question the naturalness of the genus and Tryon and Tryon (1982) put it in an expanded *Dryopteris*. However, the genus has general acceptance among world pteridologists (Pichi-Sermolli, 1977; Fraser-Jenkins, 1984, 1986; Jarrett, 1985; Gibby *et al.*, 1992; Iwatsuki, 1992; Nakaike, 1992, 2001; Ammal and Bhavananda, 1993; Shieh *et al.*, 1994; Kumar *et al.*, 1998; Moran and Øllgaard, 1998; Antony *et al.*, 2000; Hsieh, 2000) though some discrepancy about the scope of the genus exists. In the present paper the author adopts the generic concept of *Arachniodes sensu* Ching (1978), leaving *Leptorumohra* (H. Itô) H. Itô, *Acrorumohra* (H. Itô) H. Itô and *Phanerophlebiopsis* Ching, three small genera closely related to *Arachniodes*, as separate genera.

Due to the different criteria used to define species, it is very difficult to provide an exact number of species in the genus worldwide. There is little doubt that most species occur in southern China. The first checklist made by Ching (1962, 1964) recorded 22 species names from China; but since then, many new taxa have been described in the Chinese literature (Anonymous,

1974; Anonymous, 1977; Ching, 1964, 1982, 1986; Ching and Wang, 1964; Ching and Wu, 1983; Ching and Zhang, 1983; Hsieh, 1983a, 1984a, 1984b, 1986, 1991a, 1991b; Ching and Liu, 1984; Wu, 1995). To date the number of names under *Arachniodes* from China has increased to nearly 130, of which 103 species names, 2 variety names, and 4 questionable species names were documented in the Chinese version of the Flora of China (Hsieh, 2000). This has made the classification and identification of the genus very difficult in China and worldwide. It is for these reasons that the current paper has been written. It is hoped that outlining what is known about the genus will aid in the further study and enumeration of the genus.

Distribution Of *Arachniodes*

In general *Arachniodes* is a pantropical genus (Proctor, 1985; Wu, 1997) and is distributed in the subtropical to tropical forest regions of the world, mostly in China and southern to southeastern Asia. Only a small number of species are found in Central America. About 11 species are listed by Ching (1962), Proctor (1985), and Moran and Øllgaard (1998); but only 4 species are accepted by Nakaike (2001) who excluded three African and one Australian species (Ching, 1962; Gibby *et al.*, 1992; Nakaike, 2001) in *Polystichopsis* (J. Sm.) Holttum. A comparison of Japanese ferns (Kurata, 1962; Nakaike 1975; 1992; Iwatsuki, 1992) revealed that China and Japan have the greatest species diversity as well as the most species in common. The present-day distribution of *Arachniodes* is centered in the Sino-Japan region, not the Sino-Himalayan region (Ching, 1962; Wu and Ching, 1991).

In China this genus mainly occurs along the drainage area of the Yangtze and southern provinces. Its northern boundary does not exceed that of the subtropical area, to about 34°N, except for *Arachniodes exilis*, which extends northward beyond 36°N in Shandong province (Li, 1990); its western boundary is in southeastern Tibet (95°E). Most species are concentrated in southwestern and southeastern China and grow at altitudes lower than 2000 m; a few species can reach an altitude higher than 2700 m.

Taxonomic Criteria

The taxonomy of *Arachniodes* is complicated by its decompound fronds and multiple, minor morphological changes in almost all species. For a fern student who studies herbarium specimens only, it is difficult to identify species correctly. Most herbarium sheets consist merely of fronds without an attached rhizome, and without habit descriptions. The latter is important in this genus as will be discussed below. The majority of new names in the genus have been described on the basis of subtle differences in shape and other minor variations of the frond. This has led to a misleading comparison of species and has contributed to the creation of many synonyms. The most dangerous of all is the new taxa being published that are based only on single collections. For example, most of the 63 names described by Ching (1986) were only

accompanied by one cited collection and one or several duplicate sheets deposited in PE and other herbaria in China; the same is true for most of Hsieh's (1983a, 1984a, 1984b, 1986, 1991a) descriptions.

By examining more than 1250 collections of specimens in herbaria (CDBI, CTC, HITBC, KUN, PE, PYU, SZ, WNU, WUK, YAF, and some Japanese plants borrowed from TNS) and through field observations of habit in Yunnan, Sichuan, Guizhou, western Hunan and Hubei, southern Shaanxi, southern Gansu, southeastern Tibet as well as Chongqing Municipality, the author has found that the most stable and useful characters in this genus are rhizome habit and scale type. The rhizome habit can be categorized as either ascending or creeping (either short or long). The rhizome scales of most species are more or less lanceolate in shape, entire or sometimes with teeth on the margin. However, the scales found in *Arachniodes globisora* and *A. amoena* are quite specialized as will be noted below. Other useful characters include frond scales or indument, the degree of division of the lamina and each level of segmentation, shape of the lamina apex or that of the basal pinnae, shape and dissection of the ultimate pinnules, texture and luster of lamina, position of sori on the ultimate segments, and various aspects regarding the indusium. Some of the most unreliable features are the size of the frond, lamina and pinnae (especially in young fronds); the angle between rachis and the pinnae rachides; and the distribution of sori on the lamina. These characters should not be used as the sole basis for defining species. Moreover, slight to obvious morphological differences between the sterile and the fertile fronds do occur in most species, of which an extreme example is *Arachniodes dimorphophylla*.

Based on these findings, species from Yunnan and Sichuan provinces have been clarified and more than 60 names have been reduced to synonymy (He and Wu, 1996; He, 1997). But for the genus *Arachniodes* as a whole in China, it is still in need of a general revision.

Subdivision of the Genus in China

A system proposed by Hsieh (1983b) divided the genus into two sections, i.e. Sect. *Cavaleria* Ching *et* Y. T. Hsieh, and Sect. *Arachniodes*. The latter was further subdivided into two subsections and 11 series. This system attaches importance solely to the position of the sorus on veinlets of the ultimate pinnules when recognizing sections. As for the recognition of subsections and series, characters such as shape of apical pinnae, degree of frond complexity, shape and size of basal pinna pairs, that of the basiscopic pinnule of basal pinnae and that of ultimate pinnules or segments, and so on are used. In the system proposed by Hsieh (2000), some closely related species or even morphological variations within one species are placed into different subsections or series; whereas species with more fundamental differences such as habit and scale character s are put together in one section. Therefore, it is necessary to make some revision and clarification of this system.

Mainly based on the habit of rhizome, characters of rhizome and stipe base scales, and the position of sori on the veinlets of the ultimate pinnules, the

revised subdivision of *Arachniodes* categorizes the genus into four sections (He and Wu, 1996). Three of the sections have ascending rhizomes, and especially some species of sect. *Globisorae* S. K. Wu *et* H. He bear nearly erect ones; whereas the majority of species in the sect *Arachniodes* have creeping rhizomes. The four sections are well distinguished on the basis of rhizome and stipe base scales as described in Table 1. Sect. *Cavaleria* is the only group in which the sori are positioned dorsally on the veinlets of the ultimate pinnules. Though some species such as *Arachniodes globisora* and *A. henryi* were described as having have dorsal posited sori, observations of specimens revealed that the sori are only occasionally dorsal and are most often terminal on the veinlets. The erection of sect. *Globisorae* and sect. *Amoenae* (Ching *et* Y. T. Hsieh) S. K. Wu *et* H. He has taken into consideration their entire geographical distribution (Table 1) as well as characters of rhizomes and scales. Moreover, plants of sect. *Amoenae* are much more glabrous above the base of stipes. Table 1 provides a comparison of the four sections of *Arachniodes* based mainly on plants from China and adjacent regions.

Relatively few species are in the first three sections, i.e. only one species in sect. *Cavaleria*, five species in sect. *Globisorae*, and two species in sect. *Amoena*. Analyses of specimens in PE indicate that the African species *A. foliosa* (C. Chr.) Schelpe is quite similar to *A. spectablis* and could be placed in sect *Globisorae* and the Central American *A. denticulata* (Sw.) Ching could be safely treated in sect. *Amoena*. There is no doubt that most species worldwide should be placed in sect. *Arachniodes*. For the Chinese plants as a whole, 50 acceptable species names enumerated in this article belong to the section *Arachniodes* though some of them are still not satisfactory. To leave the problem open and for the purpose of convenience and further comparison, ten species groups are proposed for sect. *Arachniodes* based mainly on the rhizome habit, color of stipe scales, shape and division of the lamina, shape of pinnae and texture of the frond. Table 2 provides a comparison of these ten species groups in sect. *Arachniodes* from China.

Enumeration of *Arachniodes* from China

The following is an enumeration of names belonging to the genus *Arachniodes* Blume known from China. They are arranged in alphabetical order with original reference of publication. Accepted named are accompanied by synonyms, distribution, as well as sectional and group classification. Accepted names are in bold type; synonyms are in italics. Some of the presently accepted names, those marked with an asterisk have very few specimens available and more collections are required to prove their acceptance. For the distribution in China, the provinces listed are based on specimens checked in herbaria, unless relevant literature is cited.

Arachniodes abrupta Ching, Bull. Bot. Res., Harbin 6(3):35. 1986. = ***Arachniodes chinensis***

Arachniodes acuminata Ching *et* C. H. Wang, Acta Phytotax. Sin. 9:367. 1964. = ***Arachniodes cavalerii***

Arachniodes ailaoshanensis Ching, Bull. Bot. Res., Harbin 6(3):60. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes nipponica*. SYNONYMY: *Arachniodes jingdungensis* Ching 1986. DISTRIBUTION: Central Yunnan.

Arachniodes amoena (Ching) Ching, Acta Bot. Sin. 10: 256. 1962. *Rumohra amoena* Ching, Sinensia 5: 40, pl. 1. 1934. Sect. III. *Amoenae*.–DISTRIBUTION: Yunnan (Lu and Zhang, 1994), Guizhou, Hunan, Guangxi, Guangdong, Jiangxi, Fujian, Zhejiang, and Anhui (Chen, 1985).

****Arachniodes anshunensis*** Ching *et* Y. T. Hsieh, Bull. Bot. Res., Harbin 6(3):67, pl. 8, f. 3. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes henryi*. DISTRIBUTION: Central Guizhou.

Arachniodes aristatissima Ching, Bull. Bot. Res., Harbin 6(3):1, pl. 1, f. 1. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes simplicior*. DISTRIBUTION: Zhejiang (Hsieh, 2000; Ching, 1986).

Arachniodes assamica (Kuhn) Ohwi, J. Jap. Bot. 37:76. 1962. *Aspidium assamicum* Kuhn, Linnaea 36:108. 1869. Sect. IV. *Arachniodes*, Group *Arachniodes assamica*. SYNONYMY: *Arachniodes leuconeura* Ching 1986, *Arachniodes suijiangensis* Ching *et* Y. T. Hsieh 1986, *Arachniodes xinpingensis* Ching 1986, *Arachniodes yaomashanensis* Ching 1986, *Arachniodes basipinnata* (Ching) Ching *et* Y. T. Hsieh 1991. DISTRIBUTION: Sichuan, Chongqing, Yunnan, Guizhou, and Guangxi; Northern Thailand, Burma, Northeastern India and Sikkim.

****Arachniodes attenuata*** Ching, Bull. Bot. Res., Harbin 6(3):2, pl. 1, f. 2. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes simplicior*. DISTRIBUTION: Southern Yunnan (Ching, 1986; Hsieh, 2000) and Zhejiang (Zhang, 1993).

Arachniodes australis Y. T. Hsieh, Bull. Bot. Res., Harbin 11(3):27. 1991b. = ***Arachniodes caudata***

Arachniodes austro-yunnanensis Ching, Bull. Bot. Res., Harbin 6(3):3, pl. 1, f. 3. 1986. = ***Arachniodes sporadosora***

Arachniodes baiseensis Ching, Bull. Bot. Res., Harbin 6(3): 25. 1986. = ***Arachniodes cavalerii***

Arachniodes basipinnata (Ching) Ching *ex* Y. T. Hsieh, Bull. Bot. Res., Harbin 11(3):27. 1991b. = ***Arachniodes assamica***

Arachniodes calcarata Ching, Bull. Bot. Res., Harbin 6(3):30. 1986. = ***Arachniodes simplicior***

Arachniodes caudata Ching, Acta Phytotax. Sin. 9:384. 1964. *Polystichum simplicius* (Makino) Tagawa var. *majus* Tagawa, Acta Phytotax. Geobot. 1:90. 1932. Sect. IV. *Arachniodes*, Group *Arachniodes simplicior*. SYNONYMY: *Arachniodes caudata* Ching var. *kansuensis* Ching 1974, *Arachniodes kansuensis* (Ching) Y. T. Hsieh 1984b, *Arachniodes australis* Y. T. Hsieh

TABLE 1. Comparison of the four sections of *Arachniodes* mainly based on plants from China and adjacent regions

	Sect. I. *Cavaleria* Ching et Y. T. Hsieh	Sect. II. *Globisorae* S. K. Wu et H. He	Sect. III. *Amoenae* (Ching et Y. T. Hsieh) S. K. Wu et H. He	Sect. IV. *Arachnoides*
Section type	*Arachniodes cavalerii* (Christ) Ohwi	*Arachniodes globisora* (Hayata) Ching	*Arachniodes amoena* (Ching) Ching	*Arachniodes aspidiodes* Blume
Rhizome habit	Ascending	Ascending, sometimes nearly erect	Ascending	Creeping (long or short)
Scales on rhizome and on the base of stipe	Long-lanceolate (up to 2.5 cm long and 0.4 cm wide), entire; thin chartaceous; yellow-brown at the base of stipe and turn dark-brown upward	Long-linear (up to 3.0 cm long but only 0.2–0.3 cm wide), apical filiform, remotely ciliated or dentated; normally soft, tortuous and curled; reddish-brown, very dense; occassionally with broader scales along adaxial side	Ovate-lanceolate (ca. 0.8 cm long and 0.3 cm wide), entire, uniform; sub-coriaceous; shinning castaneous, with luster; but glabrous and glossy from the upper part of stipe to lamina	Lanceolate, linear-lanceolate, ovate or ovate lanceolate, and subulate (in general not exceed 1.0 cm long and 0.3 cm wide), entire or ciliated; soft to stiff chartaceous; often brown, reddish-brown, yellowish-brown, dark-brown or even coal black
Sori position	Dorsal on veinlets and close to the costa of the ultimate pinnule	Terminal on veinlets, occassional dorsal on veinlets of the ultimate pinnule	Terminal on veinlets	Terminal on veinlets, occasional dorsal on veinlets of the ultimate pinnule
Species	Only *Arachniodes cavalerii*	*Arachniodes globisora, A. spectabilis, A. gigantea, A. grossa,* and *A. fengii*	*Arachniodes amoena*, and *A. tonkinensis*	Ca. 50 species, which are put into 10 specie groups for further comparison (see table 2)
Distribution	South China; North Thailand and Japan	Mostly in south Yunnan and bordering region such as Vietnam and North Thailand, only *A. globisora* distributes to Taiwan, and *A. grossa* to Hainan	*A. amoena* widely distributes in South China, from Yunnan to Zhejiang; while *A. tonkinensis* only occurs in South Yunnan and Northern Vietnam	Widely distributes in Southern China, abundant along the Yangtze drainage area and southwards.

TABLE 1. Continued.

	Sect. I. *Cavaleria* Ching et Y. T. Hsieh	Sect. II. *Globisorae* S. K. Wu et H. He	Sect. III. *Amoenae* (Ching et Y. T. Hsieh) S. K. Wu et H. He	Sect. IV. *Arachnoides*
Literature cited	Bull. Bot. Res., Harbin 3(2): 77, 1983; Y. T. Hsieh in S. G. Wu, Fl. Reip. Pop. Sin. 5(1): 26, 2000, p.p., i.e. excl. spp. *Arachniodes globisora* (Hayata) Ching et *A. spinoserrulata* Ching; H. He & S. K. Wu, Acta Bot. Yunnan, 18(1): 57, 1996.	Acta Bot. Yunnan. 18(1): 57, 1996,— *Arachniodes* sect. *Cavaleria* auct.: Y. T. Hsieh in S. K. Wu, Fl. Reip. Pop. Sin. 5(1): 26, 2000, p.p.— *Arachniodes* ser. *Falcatae* auct.: Y. T. Hsieh, l. c. 52, 2000, p.p.—*Arachniodes* ser. *Festinae* auct.: Y. T. Hsieh, l. c. 77, 2000, p.p.	Acta Bot. Yunnan. 18(1): 58, 1996.— *Arachniodes* ser. *Amoenae* Ching et Y. T. Hsieh ex Y. T. Hsieh, Bull. Bot. Res., Harbin 3(2): 77, 1983; Y. T. Hsieh in S. G. Wu, Fl. Reip. Pop. Sin. 5(1): 44, 2000, p.p.	

TABLE 2. Characteristic comparison of the proposed 10 species groups of *Arachniodes* from China

Species group	Rhizome habit	Scale shape and color at the base of stipe	Lamina shape and degree of division	Shape of pinna	Texture of frond
Arachniodes assamica group	Short creeping, thick and fleshy	Lanceolate (ca. 5–8 mm long and 1–3 mm wide); entire; brown, soft	Ovate or long deltoid, with obvious acuminate apical pinna; 2-pinnate, 3-pinnatifid, occasionally 3-pinnate	Lateral pinnae normally deltoid-lanceolate	Thin chartaceous to subcoriaceous
Arachniodes coniifolia group	Short creeping, and lignified	Linear to ovate lanceolate; dark brown to total coal black; relatively dense along stipe and rachis	Ovate, apical acuminate; completely 4-pinnate	Basal pinnae deltoid to deltoid lanceolate; upper pinnae broad lanceolate	Herbaceous and thin
Arachniodes dimorphophylla group	Short creeping	Linear-lanceolate (ca. 10 mm long); entire; reddish-brown	Deltoid-lanceolate apex caudate acuminate; 2–3-pinnate; obvious dimorphophyllous	Often lanceolate	Subcoriaceous
Arachniodes exilis group	Long creeping and slender, fronds far apart	Lanceolate to linear lanceolate; reddish-brown to dark brown; soft	Broad ovate to pentagonal, apical abbreviate to caudate or slightly deltoid; often 3-pinnate	Basal pinnae long deltoid and prolonged basiscopically; the upper pinnae lanceolate	Chartaceous
Arachniodes festina group	Very short	Lanceolate; brown to dark brown; apex ciliate; firm	Oblong to lanceolate; 3–4 pinnate	Basal pinnae deltoid; the upper pinnae lanceolate	Thin herbaceous

TABLE 2. Continued.

Species group	Rhizome habit	Scale shape and color at the base of stipe	Lamina shape and degree of division	Shape of pinna	Texture of frond
Arachniodes henryi group	Short creeping, thick and fleshy	Lanceolate to ovate-lanceolate; brown or yellowish brown; entire to ciliate; soft	Ovate deltoid to broad deltoid, apex acuminate or abbreviate; 3–4 pinnate, occasionally 5-pinnate	Basal pinnae ovate deltoid; the upper pinnae lanceolate	Thin herbaceous to chartaceous
Arachniodes nipponica group	Short creeping, thick and slightly fleshy	Ovate lanceolate; brown or yellowish brown; entire; soft	Oblong to ovate, apex lightly abbreviate and acuminate; 3-pinnate	Basal pinnae oblong; the upper pinnae lanceolate pinnules, linear lanceolate	Herbaceous to chartaceous
Arachniodes rhomboidea group	Short creeping, thick and slightly fleshy	Ovate lanceolate, sometimes subulate or linear lanceolate; brown or yellowish brown	Long ovate to broad ovate, with obvious caudate apical pinna; 2–3 pinnate	Basal pinnae hastate with prolonged basiscopic	Chartaceous to subcoriaceous
Arachniodes simplicior group	Short creeping, and lignified	Lanceolate, linear lanceolate, or subulate; often ciliate; brown to reddish brown	Ovate deltoid to ovate pentagonal; apex acuminate or with distinct caudate apical pinna; 2–3 pinnate, occasionally 4-pinnate	Basal pinnae very hastate to lanceolate, with many intermediate states	Chartaceous to subcoriaceous
Arachniodes speciosa group	Short creeping and lignified	Lanceolate to ovate lanceolate; often ciliate or dentate; brown to dark brown	Ovate to ovate pentagonal; apex acuminate; 3–4 pinnate; more or less dimorphophyllous between fronds or pinnae	Basal pinnae deltoid ovate; upper pinnae oblong lanceolate	Chartaceous to subcoriaceous

1991b. DISTRIBUTION: Southern Gansu, Sichuan, Chongqing, Hunan, Yunnan, Guizhou, Guangxi, Guangdong, and Zhejiang; Japan.

Arachniodes caudata Ching var. *kansuensis* Ching, Fl. Tsinling. 2:231. 1974. = ***Arachniodes caudata*** (??)

Arachniodes caudifolia Ching *et* Y. T. Hsieh, Bull. Bot. Res., Harbin 4(2):104. 1984b. = ***Arachniodes hekiana***

Arachniodes cavalerii (Christ) Ohwi, J. Jap. Bot. 37:76. 1962. *Aspidium cavalerii* Christ, Bull. Geogr. Bot. Mans. 13:116. 1904. Sect. I. *Cavaleria*. SYNONYMY: *Arachniodes acuminata* Ching *et* C. H. Wang 1964, *Arachniodes obtusiloba* Ching *et* C. H. Wang 1964, *Arachniodes pseudo-cavalerii* Ching 1964, *Arachniodes sphaerosora* (Tagawa) Ching 1965, *Arachniodes baiseensis* Ching 1986, *Arachniodes guangxiensis* Ching 1896, *Arachniodes triangularis* Ching 1986. DISTRIBUTION: Yunnan, Guizhou, Hunan, Jiangxi, Anhui (Chen, 1985), Guangxi, Guangdong, Hainan, Fujian, and Zhejiang (Zhang, 1993); Japan and Northern Thailand.

Arachniodes centro-chinensis Ching, Fl. Tsinling. 2:229. 1974. = ***Arachniodes simulans***

Arachniodes chinensis (Rosenst.) Ching, Acta Bot. Sin. 10:257. 1962. *Polystichum amabile* (Blume) J. Sm. var. *chinense* Rosenst., Repert. Sp. Nov. 13:130. 1914. Sect. IV. *Arachniodes*, Group *Arachniodes simplicior*. SYNONYMY: *Arachniodes yaoshanensis* (Y. C. Wu) Serizawa 1973, *Arachniodes abrupta* Ching 1986, *Arachniodes costulisora* Ching 1986, *Arachniodes damiaoshanensis* Y. T. Hsieh 1986, *Arachniodes nibashanensis* Y. T. Hsieh 1986. DISTRIBUTION: Sichuan, Chongqing, Hunan, Yunnan, Guizhou, Guangxi, Guangdong, Hainan, Jiangxi, Fujian, and Zhejiang; Thailand, Indonesia and Malaysia.

Arachniodes chingii Y. T. Hsieh, Bull. Bot. Res., Harbin 6(4): 4. 1986. = ***Arachniodes simulans***

Arachniodes coniifolia (T. Moore) Ching, Acta Bot. Sin. 10:257. 1962. *Lastrea coniifolia* T. Moore, Ind. Fil. 88. 1857. Sect. IV. *Arachniodes*, Group *Arachniodes coniifolia*. SYNONYMY: *Arachniodes foeniculacea* Ching 1986, *Arachniodes guanxianensis* Ching 1986. DISTRIBUTION: Sichuan, Chongqing, Yunnan, and Guizhou; Nepal and Bhutan.

Arachniodes cornopteris Ching, Bull. Bot. Res., Harbin 6(3):4. 1986. = ***Arachniodes nanchuanensis***

Arachniodes costulisora Ching, Bull. Bot. Res., Harbin 6(3):62. 1986. = ***Arachniodes chinensis***

Arachniodes cyrtomifolia Ching, Bull. Bot. Res., Harbin 6(3):31. 1986. = ***Arachniodes nanchuanensis***

Arachniodes damiaoshanensis Y. T. Hsieh, Bull. Bot. Res., Harbin 6(4):6, f. 3.1986. = ***Arachniodes chinensis***

Arachniodes dayaoensis Y. T. Hsieh, Acta Bot. Yunnan. 5(1):57. 1983a. = ***Arachniodes simulans***

Arachniodes decomposita Ching, Bull. Bot. Res., Harbin 6(3):49. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes henryi*. DISTRIBUTION: Guizhou.

Arachniodes dimorphophylla (Hayata) Ching, Acta Bot. Sin. 10:257. 1962. *Polystichum dimorphophyllum* Hayata, Mater. Fl. Form. 30:428. 1911. Sect. IV. *Arachniodes*, Group *Arachniodes dimorphophylla*. DISTRIBUTION: Taiwan (Ching, 1962); Japan.

Arachniodes elevata Ching, Bull. Bot. Res., Harbin 6(3):40. 1986. = ***Arachniodes simulans***

Arachniodes emeiensis Ching, Bull. Bot. Res., Harbin 6(3):5, pl. 1, f. 4. 1986. = ***Arachniodes sporadosora***

Arachniodes erythrosora Ching, Bull. Bot. Res., Harbin 6(3):42. 1986. = ***Arachniodes festina***

Arachniodes exilis (Hance) Ching, Acta Bot. Sin. 10: 256.1962. *Aspidium exilis* Hance, J. Bot. 21:268. 1883. Sect. IV. *Arachniodes*, Group *Arachniodes exilis*. SYNONYMY: *Arachniodes heyuanensis* Ching 1986, *Arachniodes fengyangshanensis* Ching *et* C. F. Zhang *ex* Y. T. Hsieh 1991a. DISTRIBUTION: Hunan, Henan, Shandong, Anhui, Guangxi, Guangdong, Jiangxi, Fujian, Taiwan, Jiangsu, and Zhejiang; Japan.

Arachniodes falcata Ching, Bull. Bot. Res., Harbin 6(3):7, pl. 2, f. 1. 1986. = ***Arachniodes nanchuanensis***

Arachniodes fengii Ching, Bull. Bot. Res., Harbin 6(3):8, pl. 2, f. 2. 1986. Sect. II. *Globisorae*. DISTRIBUTION: Southern Yunnan bordering Vietnam (very rare).

Arachniodes fengyangshanensis Ching *et* C. F. Zhang *ex* Y. T. Hsieh, Bull. Bot. Res., Harbin 11(2):2. 1991a. = ***Arachniodes exilis***

Arachniodes festina (Hance) Ching, Acta Bot. Sin. 10:257. 1962. *Aspidium festinum* Hance, J. Bot. 269. 1883. Sect. IV. *Arachniodes*, Group *Arachniodes festina*. SYNONYMY: *Arachniodes erythrosora* Ching 1986. DISTRIBUTION: Guangxi, Guangdong, Taiwan, Fujian (Editorial Group of the Flora of Fujian, 1991), and Zhejiang (Zhang, 1993).

Arachniodes foeniculacea Ching, Bull. Bot. Res., Harbin 6(3):45. 1986. = ***Arachniodes coniifolia***

Arachniodes fujiangensis Ching, Bull. Bot. Res., Harbin 6(3):29, pl. 6, f. 2. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes simplicior*. DISTRIBUTION: Chongqing, Guizhou, Jiangxi, Fujian, and Zhejiang.

Arachniodes futeshanensis Y. T. Hsieh, Bull. Bot. Res., Harbin 6(4):5. 1986. = ***Arachniodes sporadosora***

Arachniodes gigantea Ching, Bull. Bot. Res., Harbin 6(3):66. 1986. Sect. II. *Globisorae*. DISTRIBUTION: Southern (bordering Northern Vietnam) and Western Yunnan.

Arachniodes gijiangensis Ching, Bull. Bot. Res., Harbin 6(3):33. 1986. = ***Arachniodes nanchuanensis***

Arachniodes gizushanensis Ching, Bull. Bot. Res., Harbin 6(3):41. 1986. = ***Arachniodes simulans***

Arachniodes globisora (Hayata) Ching, Acta Phytotax. Sin. 9:383. 1964. *Polystichum globisorum* Hayata, Icon. Pl. Form. 4:139, f. 131. 1914. Sect. II. *Globisorae*. SYNONYMY: *Arachniodes guangnanensis* Y. T. Hsieh 1984, *Arachniodes maguanensis* Ching *et* Y. T. Hsieh 1986, *Arachniodes spinuserrulata* Ching 1986, *Arachniodes menglianensis* Y. T. Hsieh 1991a. DISTRIBUTION: Yunnan (abundant in the southern counties) and Taiwan; Northern Thailand.

Arachniodes gongshanensis Ching *et* Y. T. Hsieh, Bull. Bot. Res., Harbin 6(3):68, pl. 8, f. 4. 1986. = ***Arachniodes simulans***

Arachniodes gradata Ching, Bull. Bot. Res., Harbin 6(3):39. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes simplicior*. DISTRIBUTION: Zhejiang.

Arachniodes grossa (Tard. *et* C. Chr.) Ching, Acta Bot. Sin. 10:257. 1962. *Rumohra grossa* Tard. *et* C. Chr., Notul. Syst. (Paris) 7(2):85. 1938. Sect. II. *Globisorae*. DISTRIBUTION: Hainan and Southern Guangdong; Northern Vietnam.

Arachniodes guangnanensis Y. T. Hsieh, Bull. Bot. Res., Harbin 4(2):106, f. 3. 1984b. = ***Arachniodes globisora***

Arachniodes guangtongensis Ching, Bull. Bot. Res., Harbin 6(3):58, pl. 8, f. 1. 1986. = ***Arachniodes sporadosora***

Arachniodes guangxiensis Ching, Bull. Bot. Res., Harbin 6(3):27. 1986. = ***Arachniodes cavalerii***

Arachniodes guanxianensis Ching, Bull. Bot. Res., Harbin 6(3):50. 1986. = ***Arachniodes coniifolia***

Arachniodes hainanensis (Ching) Ching, Acta Bot. Sin. 10:258. 1962. *Rumohra hainanensis* Ching, Sinensia 5:44. 1934. Sect. IV. *Arachniodes*, Group *Arachniodes dimorphophylla*. DISTRIBUTION: Hainan.

Arachniodes hekiana Kurata, J. Geobot. 13:99. 1965. Sect. IV. *Arachniodes*, Group *Arachniodes rhomboidea*. SYNONYMY: *Arachniodes rhomboidea* var. *sinica* Ching 1964, *Arachniodes caudifolia* Ching *et* Y. T. Hsieh 1984b. DISTRIBUTION: Sichuan, Chongqing, Hunan, Anhui, Yunnan, Guizhou, Guangxi, Guangdong, Fujian, and Zhejiang; Japan.

Arachniodes hekouensis Ching, Bull. Bot. Res., Harbin 6(3):57, pl. 7, f. 4. 1986. = ***Arachniodes jinpingensis***

Arachniodes henryi (Christ) Ching, Acta Bot. Sin. 10:258. 1962. *Polystichum henryi* Christ, Not. Syst. I. 36. 1909. Sect. IV. *Arachniodes*, Group *Arachniodes henryi*. DISTRIBUTION: Southern Yunnan; Northern Vietnam, Thailand and Burma.

Arachniodes heyuanensis Ching, Bull. Bot. Res., Harbin 6(3):9, pl. 2, f. 3. 1986. = ***Arachniodes exilis***

****Arachniodes huapingensis*** Ching, Bull. Bot. Res., Harbin 6(3):53, pl. 7, f. 2. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes speciosa*. DISTRIBUTION: Guangxi.

Arachniodes hunanensis Ching, Bull. Bot. Res., Harbin 6(3):10, pl. 2, f. 4. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes rhomboidea*. DISTRIBUTION: Hunan.

****Arachniodes hupingshanensis*** S. F. Wu *in* W. T. Wang, Keys Vasc. Pl. Wuling Mount. 572. 1995. Sect. IV. *Arachniodes*, Group *Arachniodes henryi*. DISTRIBUTION: Hunan (Wu, 1995).

****Arachniodes ishingensis*** Ching *et* Y. T. Xie, Acta Phytotax. Sin. 22(2):161, pl. 1, f. 2. 1984a. Sect. IV. *Arachniodes*, Group *Arachniodes exilis*. DISTRIBUTION: Jiangsu and Zhejiang.

****Arachniodes jiangxiensis*** Ching, Bull. Bot. Res., Harbin 6(3):43. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes henryi*. DISTRIBUTION: Jiangxi.

Arachniodes jinfushanensis Ching, Bull. Bot. Res., Harbin 6(3):11, pl. 3, f. 1. 1986. = ***Arachniodes simulans***

Arachniodes jingdungensis Ching, Bull. Bot. Res., Harbin 6(3):64. 1986. = ***Arachniodes ailaoshanensis***

Arachniodes jinpingensis Y. T. Hsieh, Acta Bot. Yunnan. 5(1):55, f. 1. 1983a. Sect. IV. *Arachniodes*, Group *Arachniodes simplicior*. SYNONYMY: *Arachniodes valida* Y. T. Hsieh 1983a, *Arachniodes hekouensis* Ching 1986, *Arachniodes mengziensis* Ching 1986. DISTRIBUTION: Southern Yunnan.

Arachniodes jiulunshanensis Ching, Bull. Bot. Res., Harbin 2(2):67. 1982. = ***Arachniodes simplicior***

Arachniodes kansuensis (Ching) Y. T. Hsieh, Bull. Bot. Res., Harbin 4(2): 109. 1984b. = ***Arachniodes caudata***

****Arachniodes lanceolata*** Y. T. Hsieh, Bull. Bot. Res., Harbin 11(2):4. 1991a. Sect. IV. *Arachniodes*, Group *Arachniodes simplicior*. DISTRIBUTION: Sichuan (Mt. Emei).

Arachniodes leuconeura Ching, Bull. Bot. Res., Harbin 6(3):12, pl. 3, f. 2. 1986. = ***Arachniodes assamica***

Arachniodes liyangensis Ching *et* Y. C. Lan, Fl. Jiangsu. 1:63, 466. 1977. Sect. IV. *Arachniodes*, Group *Arachniodes simplicior*. DISTRIBUTION: Jiangsu (Anonymous, 1977) and Anhui (Chen, 1985).

Arachniodes longipinna Ching, Bull. Bot. Res., Harbin 6(3):13, pl. 3, f. 3. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes simplicior*. DISTRIBUTION: Guangxi.

****Arachniodes lushanensis*** Ching, Bull. Bot. Res., Harbin 6(3):61. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes exilis*. DISTRIBUTION: Jiangxi.

Arachniodes lushuiensis Y. T. Hsieh, Bull. Bot. Res., Harbin 4(2):108, f. 4. 1984b. = ***Arachniodes simulans***

Arachniodes maguanensis Ching *et* Y. T. Hsieh, Bull. Bot. Res., Harbin 6(4):2, f. 2. 1986. = ***Arachniodes globisora***

****Arachniodes maoshanensis*** Ching, Bull. Bot. Res., Harbin 6(3):54, pl. 7, f. 3. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes exilis*. DISTRIBUTION: Zhejiang.

Arachniodes menglianensis Y. T. Hsieh, Bull. Bot. Res., Harbin 11(2):3. 1991a. = ***Arachniodes globisora***

Arachniodes mengziensis Ching, Bull. Bot. Res., Harbin 6(3):14, pl. 3, f. 4. 1986. = ***Arachniodes jinpingensis***

****Arachniodes michelii*** (H. Lév.) Ching *ex* Y. T. Hsieh, Bull. Bot. Res., Harbin 11(3):27. 1991b. *Dryopteris michelii* Lév., Fl. Kouy-Tscheou. 493. 1915. Sect. IV. *Arachniodes*, Group *Arachniodes exilis*. DISTRIBUTION: Guizhou and Hunan (Hsieh, 2000).

Arachniodes multifida Ching, Bull. Bot. Res., Harbin 6(3):15, pl. 4, f. 1. 1986. = ***Arachniodes sporadosora***

Arachniodes nanchuanensis Ching *et* Z. Y. Liu, Bull. Bot. Res., Harbin 4(4):21, f. 50. 1984. Sect. IV. *Arachniodes*, Group *Arachniodes simplicior*. SYNONYMY: *Arachniodes cornopteris* Ching 1986, *Arachniodes cyrtomifolia* Ching 1986, *Arachniodes falcata* Ching 1986, *Arachniodes gijiangensis* Ching 1986, *Arachniodes semifertilis* Ching 1986. DISTRIBUTION: Chongqing (very abundant), Sichuan, and Yunnan.

Arachniodes nanqingensis Ching, Bull. Bot. Res., Harbin 6(3):38. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes simplicior*. DISTRIBUTION: Fujian.

Arachniodes neoaristata Ching, Bull. Bot. Res., Harbin 6(3):34, pl. 6, f. 3. 1986. = ***Arachniodes sporadosora***

Arachniodes nibashanensis Y. T. Hsieh, Bull. Bot. Res., Harbin 6(4):7, f. 4. 1986. = ***Arachniodes chinensis***

Arachniodes nigrospinosa (Ching) Ching, Acta Bot. Sin. 10:258. 1962. *Polystichum nigrospinosum* Ching, Bull. Fan Mem. Inst. Biol. Bot. Ser. 2:191, f. 6. 1931. Sect. IV. *Arachniodes*, Group *Arachniodes coniifolia*. DISTRIBUTION: Guizhou, Guangxi, Guangdong, and Taiwan.

Arachniodes nipponica (Rosenst.) Ohwi, J. Jap. Bot. 37:76. 1962. *Polystichum nipponicum* Rosenst., Repert. Sp. Nov. 13:190. 1914. Sect. IV. *Arachniodes*, Group *Arachniodes nipponica*. DISTRIBUTION: Sichuan, Chongqing, Yunnan, Guizhou, Hunan, Guangdong, Jiangxi, and Zhejiang; Japan.

Arachniodes nitidula Ching, Bull. Bot. Res., Harbin 6(3):59, pl. 8, f. 2. 1986. = ***Arachniodes spectabilis***

Arachniodes obtusiloba Ching *et* C. H. Wang, Acta Phytotax. Sin. 9:369. 1964. = ***Arachniodes cavalerii***

Arachniodes obtusipinnula Ching *et* Y. T. Hsieh, Acta Phytotax. Sin. 22(2):160, pl. 1, f. 1. 1984a. = ***Arachniodes tonkinensis***

Arachniodes parasimplicior Ching *ex* Y. T. Hsieh, Bull. Bot. Res., Harbin 11(2):1. 1991a. = ***Arachniodes simplicior***

Arachniodes pianmaensis Ching, Bull. Bot. Res., Harbin 6(3):65. 1986. = ***Arachniodes simulans***

Arachniodes pseudo-aristata (Tagawa) Ohwi, J. Jap. Bot. 37:76. 1962. = ***Arachniodes sporadosora***

Arachniodes pseudo-assamica Ching, Bull. Bot. Res., Harbin 6(3):16, pl. 4, f. 2. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes assamica*. DISTRIBUTION: Southern Yunnan.

Arachniodes pseudo-cavalerii Ching, Acta Phytotax. Sin. 9:376. 1964. = ***Arachniodes cavalerii***

Arachniodes pseudo-hekiana Kurata, J. Geobot. 16:5. 1968. Sect. IV. *Arachniodes*, Group *Arachniodes rhomboidea*. DISTRIBUTION: Yunnan, Guangdong, and Jiangxi; Japan.

Arachniodes pseudo-longipinna Ching, Bull. Bot. Res., Harbin 6(3):17. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes simplicior*. DISTRIBUTION: Guangxi.

Arachniodes pseudo-simplicior Ching, Bull. Bot. Res., Harbin 6(3):47. 1986. = ***Arachniodes ziyunshanensis***

Arachniodes reducta Y. T. Hsieh *et* Y. P. Wu, Bull. Bot. Res., Harbin 4(2):105, f. 2. 1984b. Sect. IV. *Arachniodes*, Group *Arachniodes simplicior*. DISTRIBUTION: Zhejiang.

Arachniodes rhomboidea (Wall. *ex* C. Presl) Ching, Acta Bot. Sin. 9:384. 1964. *Polystichum rhomboideum* Wall. *ex* C. Presl, Epim. Bot. 54. 1851. Sect. IV. *Arachniodes*, Group *Arachniodes rhomboidea*. DISTRIBUTION: Sichuan, Chongqing, Hubei, Hunan, Anhui, Yunnan, Guizhou, Guangxi, Guangdong, Jiangxi, Fujian, Jiangsu, and Zhejiang; Japan, India and Nepal.

Arachniodes rhomboidea (Wall. *ex* Mett.) Ching var. *sinica* Ching, Acta Phytotax. Sin. 9:384. 1964. = ***Arachniodes hekiana***

Arachniodes semifertilis Ching, Bull. Bot. Res., Harbin 6(3):18, pl. 4, f. 3. 1986. = ***Arachniodes nanchuanensis***

****Arachniodes setifera*** Ching, Bull. Bot. Res., Harbin 6(3):52, pl. 7, f. 1. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes exilis*. DISTRIBUTION: Guangxi.

Arachniodes shuangbaiensis Ching, Bull. Bot. Res., Harbin 6(3):21, pl. 5, f. 1. 1986. = ***Arachniodes ziyunshanensis***

Arachniodes sichuanensis Ching, Bull. Bot. Res., Harbin 6(3):36. 1986. = ***Arachniodes sporadosora***

Arachniodes similis Ching, Bull. Bot. Res., Harbin 6(3):19, pl. 4, f. 4. 1986. = ***Arachniodes tiendongensis***

Arachniodes simplicior (Makino) Ohwi, J. Jap. Bot. 37:76. 1962. *Aspidium aristatum* var. *simplicius* Makino, Bot. Mag. Tokyo 15:65. 1901. Sect. IV.

Arachniodes, Group *Arachniodes simplicior*. SYNONYMY: *Arachniodes jiulunshanensis* Ching 1982, *Arachniodes tibetana* Ching *et* S. K. Wu 1983, *Arachniodes calcarata* Ching 1986, *Arachniodes parasimplicior* Ching *ex* Y. T. Hsieh 1991a. DISTRIBUTION: Southern Gansu, Southern Shaanxi, Henan (Hsieh, 2000), Sichuan, Chongqing, Hubei, Hunan, Anhui, Xizang (Tibet), Yunnan, Guizhou, Guangxi, Guangdong (Miao *et al.*, 1997), Jiangxi, Fujian, Jiangsu (Anonymous, 1977), and Zhejiang; Japan.

Arachniodes simulans (Ching) Ching, Acta Bot. Sin. 10:259. 1962. *Rumohra simulans* Ching, Sinensia 5:54, pl. 8. 1934. Sect. IV. *Arachniodes*, Group *Arachniodes henryi*. SYNONYMY: *Arachniodes centro-chinensis* Ching 1974, *Arachniodes dayaoensis* Y. T. Hsieh 1983a, *Arachniodes lushuiensis* Y. T. Hsieh 1984b, *Arachniodes chingii* Y. T. Hsieh 1986, *Arachniodes elevata* Ching 1986, *Arachniodes gizushanensis* Ching 1986, *Arachniodes gongshanensis* Ching *et* Y. T. Hsieh 1986, *Arachniodes jinfushanensis* Ching 1986, *Arachniodes pianmaensis* Ching 1986, *Arachniodes yunnanensis* Ching 1986. DISTRIBUTION: Southern Gansu, Southern Shaanxi, Sichuan, Chongqing, Hubei, Hunan, Jiangxi, Yunnan, and Guizhou; Bhutan.

Arachniodes sino-aristata Ching, Bull. Bot. Res., Harbin 6(3):20. 1986. = ***Arachniodes sporadosora***

Arachniodes sino-rhomboidea Ching, Bull. Bot. Res., Harbin 6(3):55. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes henryi*. DISTRIBUTION: Sichuan.

Arachniodes sparsa Ching, Bull. Bot. Res., Harbin 6(3):22, pl. 5, f. 2. 1986. = ***Arachniodes sporadosora***

Arachniodes speciosa (D. Don) Ching, Acta Bot. Sin. 10:259. 1962. *Aspidium speciosum* D. Don, Prodr. Fl. Nepal. 5. 1825. Sect. IV. *Arachniodes*, Group *Arachniodes speciosa*. DISTRIBUTION: Yunnan, Guangxi and Hainan; Sikkim, Bhutan and Nepal.–This is a questionable species. Specimens deposited in PE, PYU and KUN, which were previously identified by Ching under this name, are now typically placed under other names, most of which are synonyms of *Arachniodes sporadosora*.

Arachniodes spectabilis (Ching) Ching, Acta Bot. Sin. 10:259. 1962. *Rumohra spectabilis* Ching, Sinensia 5: 58, pl. 11. 1934. Sect. II. *Globisorae*. SYNONYMY: *Arachniodes nitidula* Ching 1986. DISTRIBUTION: Southern Yunnan; Northern Thailand, and Laos (Itô, 1974).

Arachniodes sphaerosora (Tagawa) Ching, Acta Phytotax. Sin. 10:192. 1965. = ***Arachniodes cavalerii***

Arachniodes spinu-serrulata Ching, Bull. Bot. Res., Harbin 6(3):46, pl. 6, f. 4. 1986. = ***Arachniodes globisora***

Arachniodes sporadosora (Kunze) Nakaike, Enum. Pteridophyt. Japon. Fil. 192. 1975. *Aspidium sporadosorum* Kunze, Bot. Zeit. 6: 556. 1848. Sect. IV. *Arachniodes*, Group *Arachniodes speciosa*. SYNONYMY: *Arachniodes pseudo-aristata* (Tagawa) Ohwi 1962, *Arachniodes subaristata* Ching *et* Y.

T. Hsieh 1984b, *Arachniodes austro-yunnanensis* Ching 1986, *Arachniodes emeiensis* Ching 1986, *Arachniodes futeshanensis* Y. T. Hsieh 1986, *Arachniodes guangtongensis* Ching 1986, *Arachniodes multifida* Ching 1986, *Arachniodes neo-aristata* Ching 1986, *Arachniodes sparsa* Ching 1986, *Arachniodes sichuanensis* Ching 1986, *Arachniodes sino-aristata* Ching 1986. DISTRIBUTION: Sichuan, Chongqing, Hunan, Yunnan, Guizhou, Guangxi, Jiangxi, Fujian, and Zhejiang; Japan.

Arachniodes subamoena Ching, Bull. Bot. Res., Harbin 6(3):51. 1986. = ***Arachniodes tonkinensis***

Arachniodes subaristata Ching *et* Y. T. Hsieh, Bull. Bot. Res., Harbin 4(2):103, f. 1.1984b. = ***Arachniodes sporadosora***

Arachniodes suijiangensis Ching *et* Y. T. Hsieh, Bull. Bot. Res., Harbin 6(4):1, f. 1. 1986. = ***Arachniodes assamica***

Arachniodes tibetana Ching *et* S. K. Wu in C. Y. Wu, Fl. Xizang. 1. 243, pl. 59, f. 3–4. 1983. = ***Arachniodes simplicior***

Arachniodes tiendongensis Ching *et* C. F. Zhang, Bull. Bot. Res., Harbin 3(3):9. 1983. Sect. IV. *Arachniodes*, Group *Arachniodes rhomboidea*. SYNONYMY: *Arachniodes similis* Ching 1986 DISTRIBUTION: Zhejiang and Guangdong.

Arachniodes tonkinensis (Ching) Ching, Acta Bot. Sin. 10:260. 1962. *Rumohra tonkinensis* Ching, Sinensia 5:52. 1934. Sect. III. *Amoenae*. Synonymy: *Arachniodes obtusipinnula* Ching *et* Y. T. Hsieh 1984a, *Arachniodes subamoena* Ching 1986. DISTRIBUTION: Southern Yunnan; Northern Vietnam.

Arachniodes triangularis Ching, Bull. Bot. Res., Harbin 6(3):26, pl. 6, f.1.1986. = ***Arachniodes cavalerii***

Arachniodes valida Y. T. Hsieh, Acta Bot. Yunnan. 5(1):56. 1983a. = ***Arachniodes jinpingensis***

****Arachniodes wulingshanensis*** S. F. Wu *in* W. T. Wang, Keys Vasc. Pl. Wuling Mount. 572, t. 5. 1995. Sect. IV. *Arachniodes*, Group *Arachniodes assamica*. DISTRIBUTION: Hunan (Wu, 1995).

Arachniodes xinpingensis Ching, Bull. Bot. Res., Harbin 6(3):23, pl. 5, f. 3. 1986. = ***Arachniodes assamica***

****Arachniodes yandangshanensis*** Y. T. Xie, Acta Phytotax. Sin. 22(2):161, pl. 1, f. 3. 1984a. Sect. IV. *Arachniodes*, Group *Arachniodes speciosa*. DISTRIBUTION: Zhejiang.

Arachniodes yaomashanensis Ching, Bull. Bot. Res., Harbin 6(3):32. 1986. = ***Arachniodes assamica***

Arachniodes yaoshanensis (Y. C. Wu) Serizawa, J. Jap. Bot. 48:219. 1973. = ***Arachniodes chinensis***

Arachniodes yinjiangensis Ching, Bull. Bot. Res., Harbin 6(3):44. 1986. Sect. IV. *Arachniodes*, Group *Arachniodes henryi*. DISTRIBUTION: Northeastern Guizhou.

Arachniodes yoshinagae (Makino) Ching, Acta Phytotax. Sin. 9:383. 1964. *Aspidium yoshinagae* Makino, Bot. Mag. Tokyo 13:57. 1899. Sect. IV. *Arachniodes*, Group *Arachniodes assamica*. DISTRIBUTION: Chongqing, Hunan; Japan.

Arachniodes yunnanensis Ching, Bull. Bot. Res., Harbin 6(3):24, pl. 5, f. 4. 1986. = ***Arachniodes simulans***

Arachniodes yunqiensis Y. T. Hsieh, Bull. Bot. Res., Harbin 6(4):3. 1986. = ***Arachniodes ziyunshanensis***

Arachniodes ziyunshanensis Y. T. Xie, Acta Phytotax. Sin. 22(2):162, pl. 1, f. 4. 1984. Sect. IV. *Arachniodes*, Group *Arachniodes simplicior*. SYNONYMY: *Arachniodes pseudo-simplicior* Ching 1986, *Arachniodes shuangbaiensis* Ching 1986, *Arachniodes yunqiensis* Y. T. Hsieh 1986. DISTRIBUTION: Chongqing, Hunan, Yunnan, and Zhejiang.

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Chromosome Behavior During Meiosis and Development of Spore Mother Cells in the Chinese Quillwort *Isoetes sinensis* T. C. Palmer (Isoetaceae)

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ABSTRACT.—Chromosome behavior during meiosis of the tetraploid *Isoetes sinensis* was observed to be similar to that of basic diploid *Isoetes* species. This observation was consistent with the previous report that polyploid species of *Isoetes* are allopolyploids. Meiosis was generally similar in both megaspore and microspore mother cells, but differences were observed. Due to the smaller size of the microspore mother cells, during prophase I, chromosomes are not as dispersed as those in megaspore mother cells. Cytomixis was observed in all stages of meiosis in microspore mother cells, but it was not seen at any stage of meiosis in megaspore mother cells. Cytomixis, lagging chromosomes, chromosome bridges, chromosome fragments, and micronuclei, observed during meiosis in *I. sinensis*, can affect sexual reproduction, but it does not appear to be a major cause of population decline in this species. The major cause of decline is habitat degradation from human disturbance. The few remaining populations of the Chinese quillwort are fragmented and the numbers of individuals contained in these populations dwindle as a result of habitat degradation. Conservation efforts are needed to save *I. sinensis* from extinction.

The Isoetaceae is an ancient family of heterosporous lycopsids. It includes one cosmopolitan genus, *Isoetes*, which contains from 130 to 350 living species (Jermy, 1990; Hickey *et al.*, 2003). Species range in habit from evergreen aquatics to ephemeral terrestrials (Taylor and Hickey, 1992). Chromosome counts of *Isoetes* range from $2n = 2x = 20$, 22 to $2n = 12x = 132$ (Manton 1950; Löve and Löve, 1977; Kott and Britton, 1980; Hickey, 1984; Bhu and Goswami, 1990; Takamiya *et al.*, 1994). For the four Chinese species, Liu *et al.* (2002) reported $2n = 22$ counts for *Isoetes hypsophila* Handel-Mazetti, *I. taiwanensis* DeVol and *I. yunguiensis* Q. F. Wang & W. C. Taylor and $2n = 44$ for *Isoetes sinensis* T. C. Palmer. He *et al.* (2002) also reported a $2n = 44$ count for *I. sinensis*.

Chromosome behavior and pairing during meiosis are related to the phylogeny and ecology of plant populations. Synapsis, crossing over, and segregation of chromosomes during meiosis affect the evolution and adaptation of taxa. In this regard, heterosporous lycopods such as *Isoetes* have evolved a unique reproductive system worthy of study. There have been few studies of chromosome behavior during meiosis in spore mother cells of *Isoetes* except for chromosome figures in metaphase I for several Indian species by Bhu and Goswami (1990) and the Japanese taxa by Takamiya *et al.* (1996).

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Therefore, the goals of this study were to: 1) describe chromosome behavior during sporogenesis in the endangered species *I. sinensis*, 2) compare meiotic behaviors in microspore and megaspore mother cells and 3) determine if there are cytological irregularities during meiosis in *I. sinensis* that could affect its reproduction.

Materials And Methods

Samples were collected from two native *Isoetes sinensis* populations, one in Songyang County, Zhejiang Province that included two subpopulations 800 m apart and another in Xiuning County, Anhui Province. Collections were made in July 2002 and the living plants were cultivated in ponds in the Wuhan Botanical Garden, Chinese Academy of Sciences. The voucher specimens were deposited in the Wuhan Botanical Garden Herbarium, Chinese Academy of Sciences (HIB). To obtain somatic chromosome figures, young leaves from the shoot apex of six plants were pretreated in 0.1% colchicine for 3 hours before fixing. To obtain meiocytes in the proper stage for meiotic analysis, young intact sporangia were harvested from 20 different plants between 20 April and 20 May 2003. Both somatic and meiotic samples were fixed in Farmer's Solution (3:1 absolute ethyl alcohol: glacial acetic acid) for 30 minutes and then placed in refrigerated 70% alcohol for at least 30 minutes. After washing with distilled water 3 times, the sporangia were transferred to a 30 mmol/L (pH 4.5) citric acid buffer containing a mixed enzyme solution of 1.5% cellulase "Onozuka" Rs (Yakult Honsha Co. Ltd, Tokyo, Japan) and 1.5% pectolyase Y23 (Sigma P3026) at 34°C for one hour. The digested tissue was then washed 3 times in distilled water for a total of 30 minutes and transferred to fresh Farmer's Solution for 30 minutes. Sporangia were opened and meiocytes dispersed in 2–3 drops of Carnoy's fluid on a microscope slide. The slide was gently flamed until dry and then stained with Giemsa at pH 7.1 for 30 minutes. Each slide was washed for several seconds and examined for figures using a Leitz compound microscope.

Results

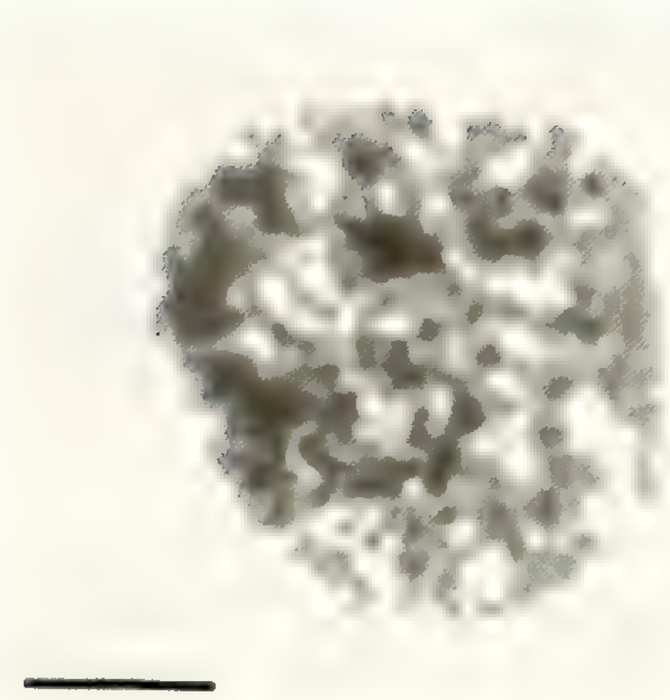
Somatic chromosome counts of *Isoetes sinensis* from both of the populations sampled were uniformly $2n = 44$. This is consistent with the previous counts by He *et al.* (2002) and Liu *et al.* (2002). The interphase nucleus in *Isoetes sinensis* is of the complex type, with heterochromatic fragments forming irregular chromocenters scattered throughout the nucleus (Fig. 1) and prophase chromosomes (Fig. 2) belonged to the interstitial type, i.e., the heterochromatic fragments and euchromatic fragments distributed with no obvious boundaries (Tanaka 1971, 1977). In metaphase, the ratio of the longest chromosome to the shortest was 1.35:1 and chromosome lengths ranged from 3.7–5.0 μm (Fig. 3). At anaphase, the centromere of each chromosome divided and the sister chromatids moved to opposite poles (Fig. 4). The mitotic divisions in a sporangium that proceeded meiosis were nearly synchronous, and the large and densely stained mitotic nuclei were obvious in the sporogenous tissue (Figs. 5–6).

The megaspore and microspore mother cells differ in their morphology. Each megaspore mother cell was ovoid to globose, 62–75 μm in diameter, with a transparent, thick perine enclosing the cell. The cytoplasm was dense, viscous, readily stained, and contained many nucleoli dispersed throughout the cell (Fig. 7). Microspore mother cells were globose, 10–12 μm in diameter, with a transparent, thin perine enclosing the cell and contained few nucleoli dispersed throughout cell (Fig. 8). A few sporangia appeared to contain both megaspore mother cells and microspore mother cells. Sporangia containing both immature megaspores and tetrads of microspores were also observed (Fig. 45). Immature megaspores were 126–129 μm in diameter, including the transparent outer perine (Figs. 44–45). Mature megaspores were 313–316 μm in diameter, including the perine. Immature microspores were 14–16 μm, ellipsoid to kidney shaped, and bilaterally symmetrical. Uniform mature microspores were 26–28 μm.

The First Meiotic Division.—In early prophase I, the filamentous chromosomes, derived from chromatin condensation, were dispersed throughout the megaspore mother cell. At leptotene, the thread-like chromosomes formed a tangled reticulum (Fig. 9). At zygotene, regions of the homologous chromosomes that had achieved synapsis were thicker than the unpaired regions. Different pairing patterns for homologous chromosomes were observed. In some cases, synapsis began simultaneously at several contact points along the length of the chromosomes. In other cases, synapsis proceeded from one end to the other. In a few cases, regions of homologous chromosomes remained unpaired indicating that some structural differences existed between the two homologs. During zygotene, we also observed a few abnormal chromosome pairing behaviors like duplication pairing and partial partner switching between two homologous chromosomes and a third chromosome (Fig. 10). Duplication pairing and partial partner switching are the two of forms of multivalent synapsis. At early pachytene, homologous chromosomes had completed bivalent formation. Except for an occasional duplicate pairing or multivalent synapsis, homologous chromosome pairing was normal (Figs. 11–12). At pachytene, the twenty-two linear bivalents became easier to distinguish as they continued to thicken (Fig. 13). The chromosomes appeared nearly equal in length and chromomeres were clearly discernible. At diplotene, chromosome chiasma occurred (Fig. 14).

During microsporogenesis, chromosome morphology, condensation, pairing, synapsis, and segregation were generally similar to that observed in megaspore mother cells (Figs. 15–16 leptotene; Figs. 17–18, zygotene; Figs. 19–20, pachytene; Fig. 21, diplotene), but there some were obvious differences. First, at prophase I, the size of the microspore mother cell constrains the spread of condensing chromosomes and hence chromosomes are more conjested than those in the megaspore mother cell (cf. Figs. 9–14 with Figs. 15–20). Second, cytomixis occurred throughout microsporogenesis (Figs. 22–26), whereas it was absent during megasporogenesis.

During diakinesis and metaphase I, bivalents were observed in megaspore mother cells and microspore mother cells (Figs. 27–28). Univalents or



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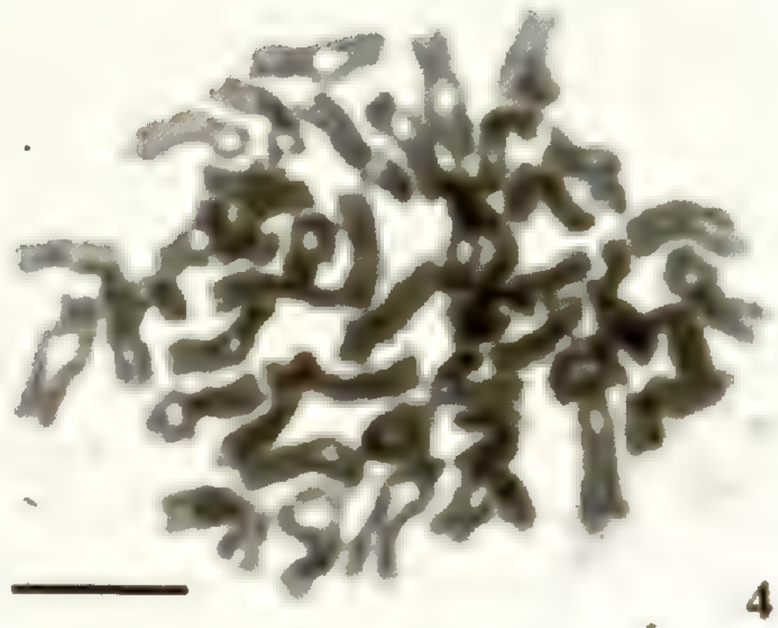

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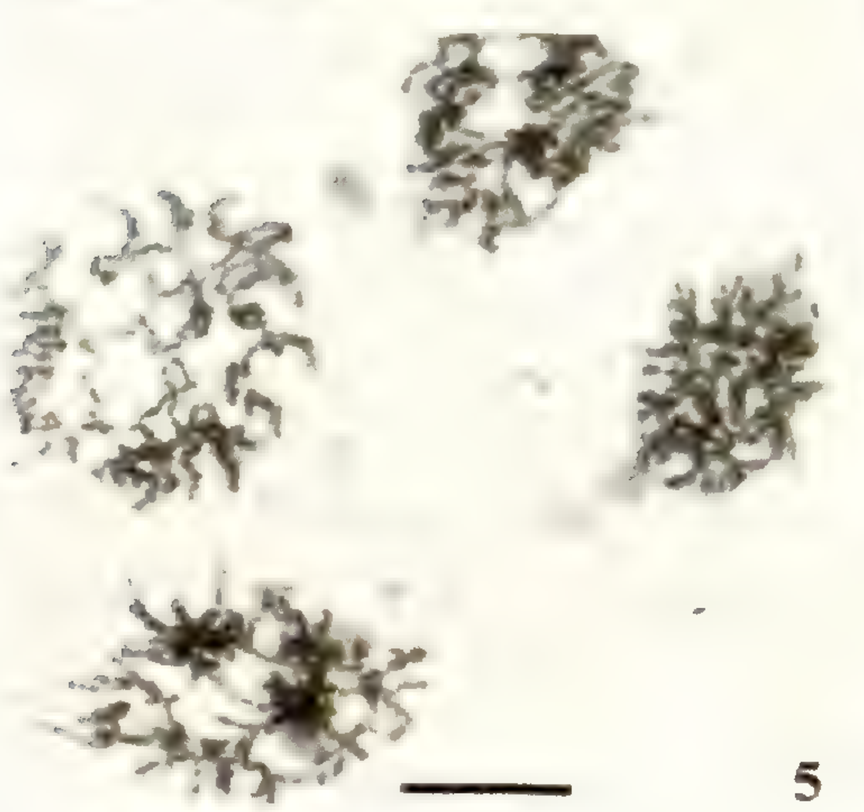

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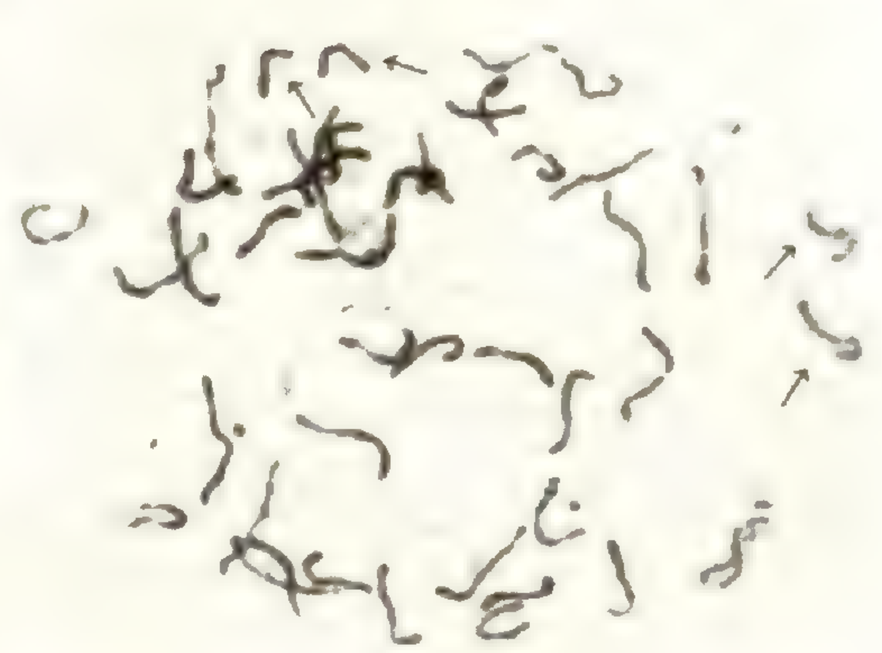

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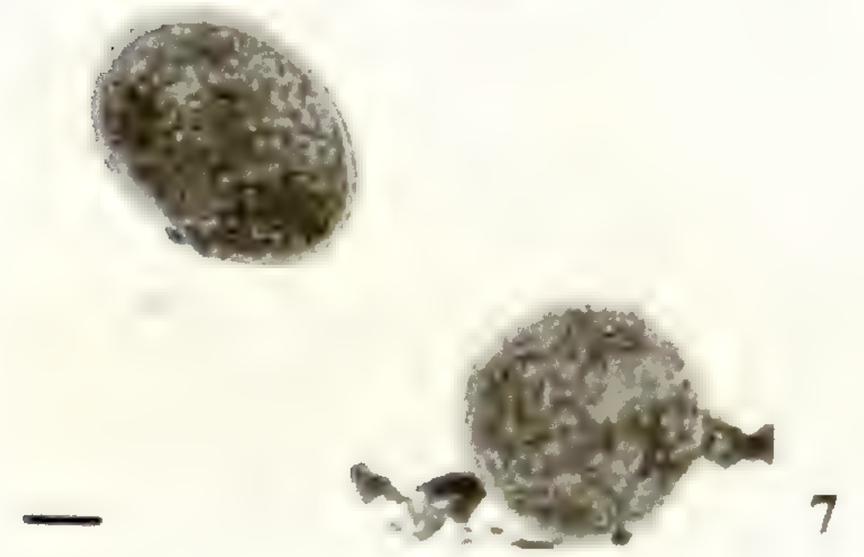

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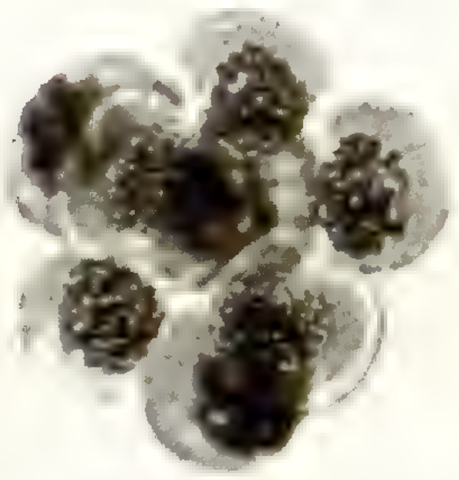

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FIGS. 1–8. Mitosis in *Isoetes sinensis*. 1–4. Somatic cells from young leaves of the shoot apex of *Isoetes sinensis*. 1. Interphase, showing heterochromatic fragments forming irregular chromocenters scattered throughout the nucleus. 2. Late prophase, showing chromosomes of interstitial type. 3. Metaphase. 4. Early anaphase, with centromere of each chromosome divided. 5–6. Chromosome figures of mitosis preceding meiosis. 5. Prophase (microspores), showing nearly synchronous mitotic divisions in a sporangium preceeding meiosis. 6. Early metaphase (microspores), showing homologous chromosomes (arrows). 7. Megaspore mother cells. 8. Microspore mother cells. Scale bars in Figs. 1 & 3 = 5 μm; Figs. 2, 4, 6 & 8 = 10 μm; Figs. 5, 7 = 20 μm.

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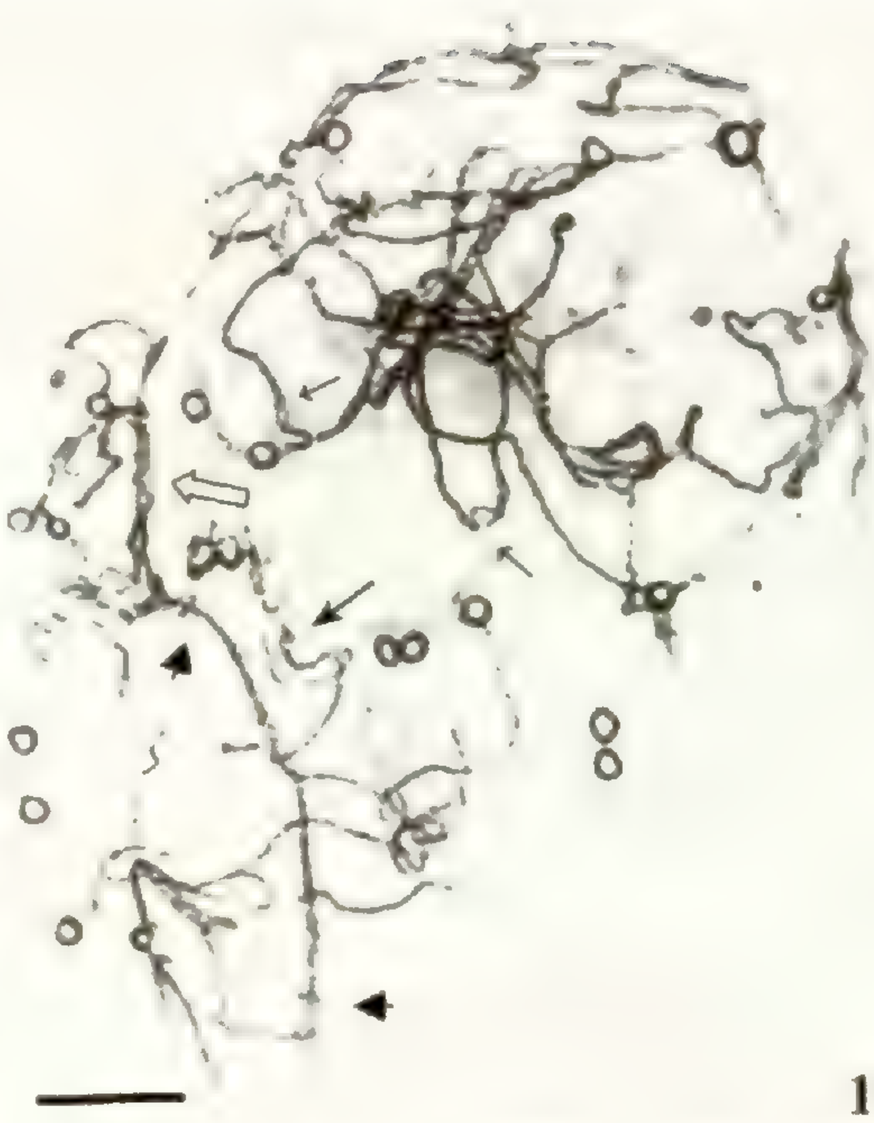

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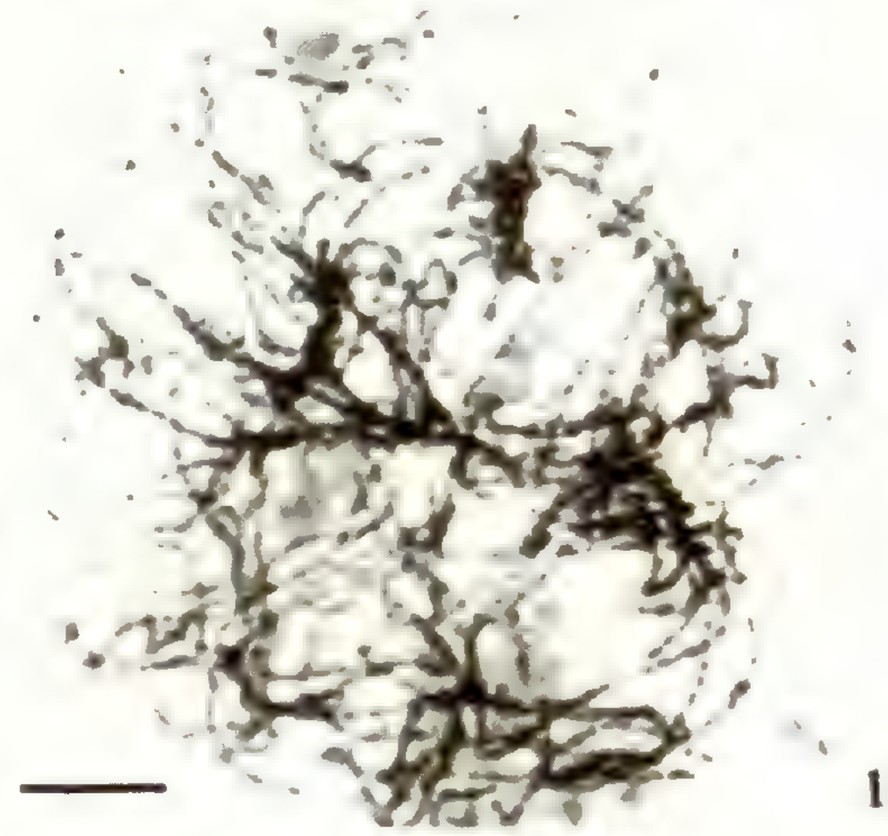

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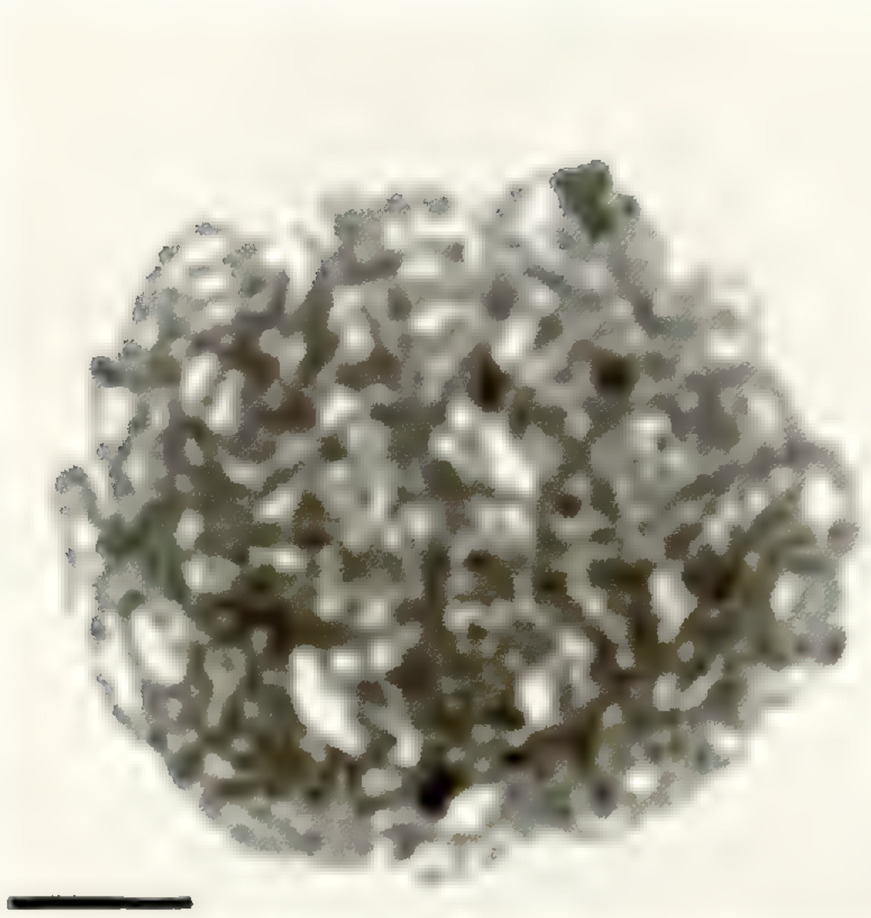
FIGS. 9–14. Megasporogenesis in *Isoetes sinensis*. 9. Leptotene, showing the thread-like chromosomes. 10. Zygotene, showing a partial partner switching (hollow arrow), acrosyndesis, i.e. chromosomes pairing end-to-end (long arrow), unpaired region (short arrow), and slight structural differences in homologous chromosome (arrow heads). 11–12. Early pachytene. 11. Multivalent synapsis (hollow arrow) and partial duplicate pairing (arrow heads). 12. Partial duplicate pairing (arrow heads). 13. Pachytene, showing the linear bivalents. 14. Diplotene, showing chiasma. Scale bars in Figs. 10 & 14 = 5 μm; Figs. 9 & 11–13 = 10 μm.



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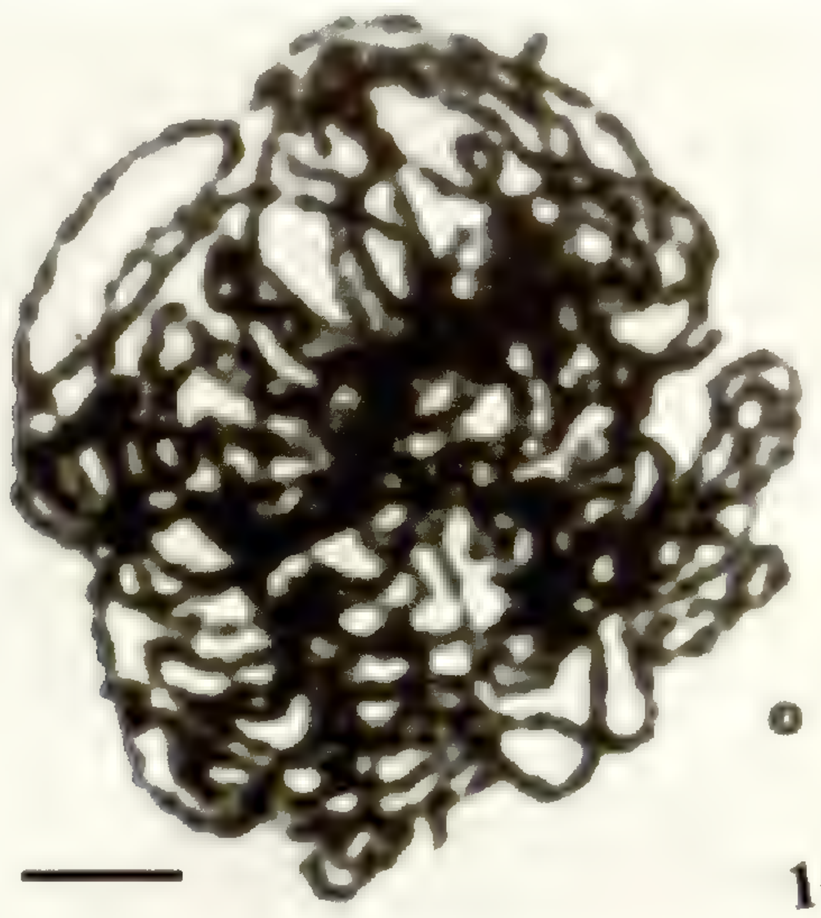

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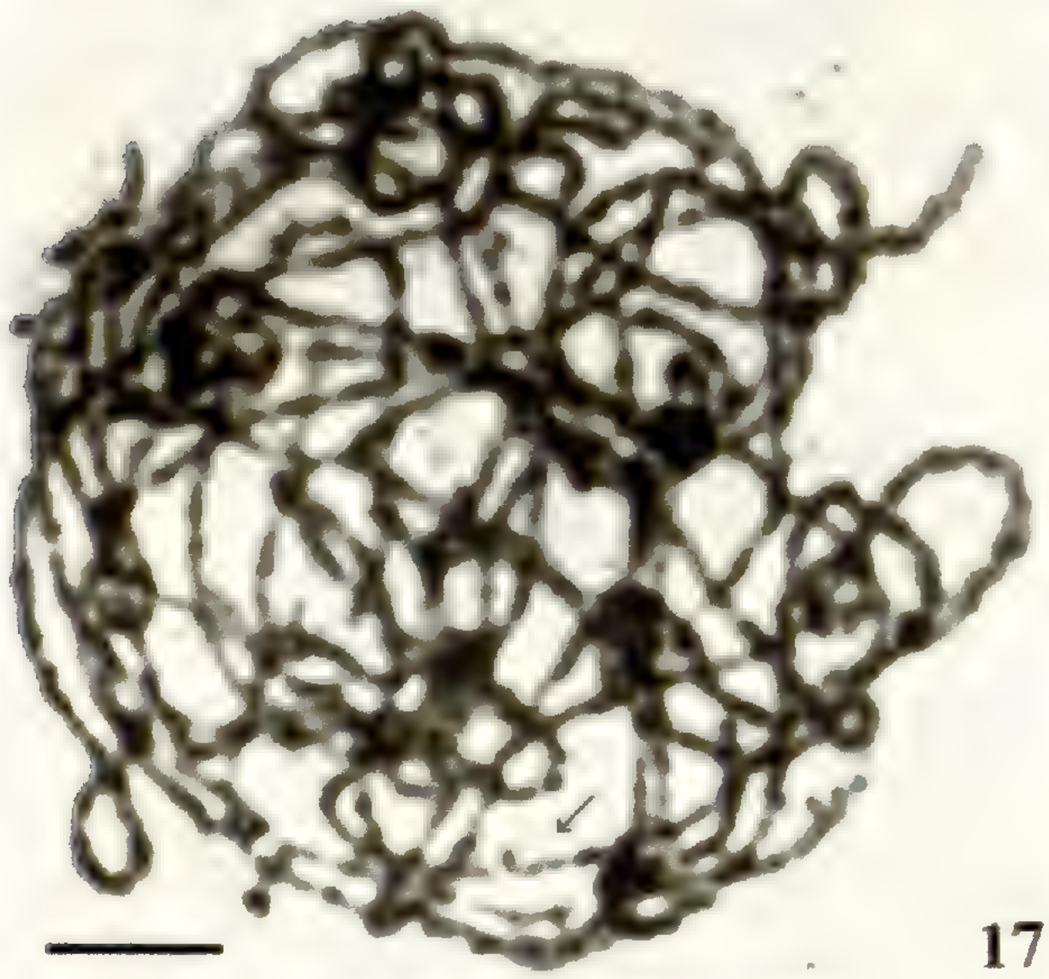

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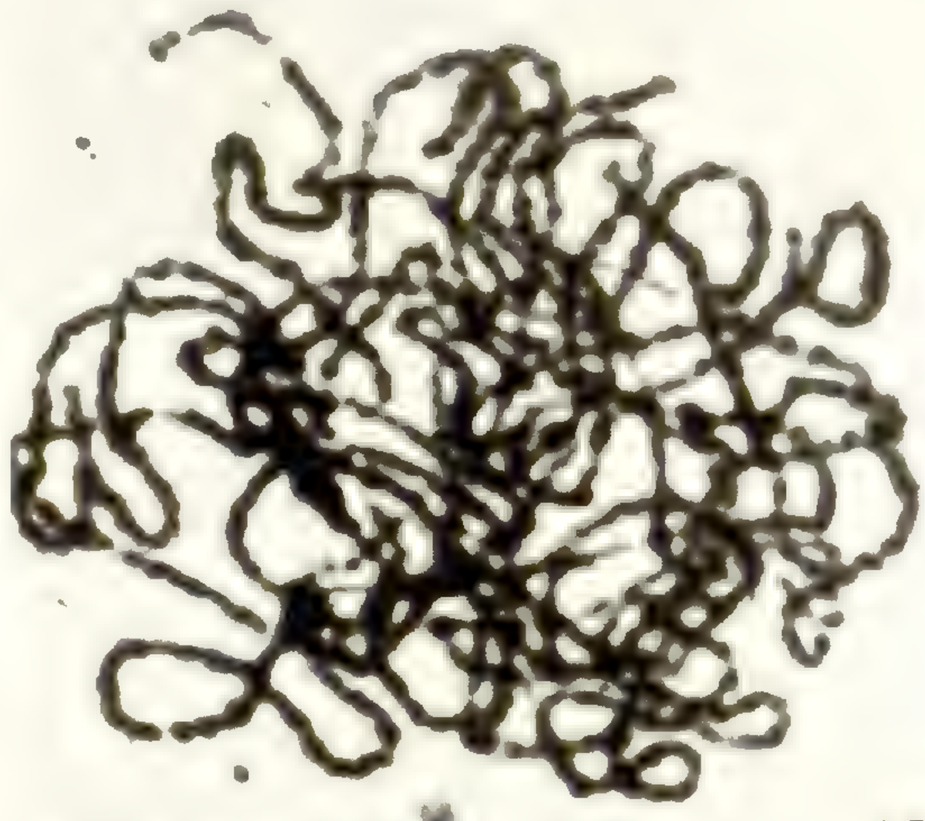

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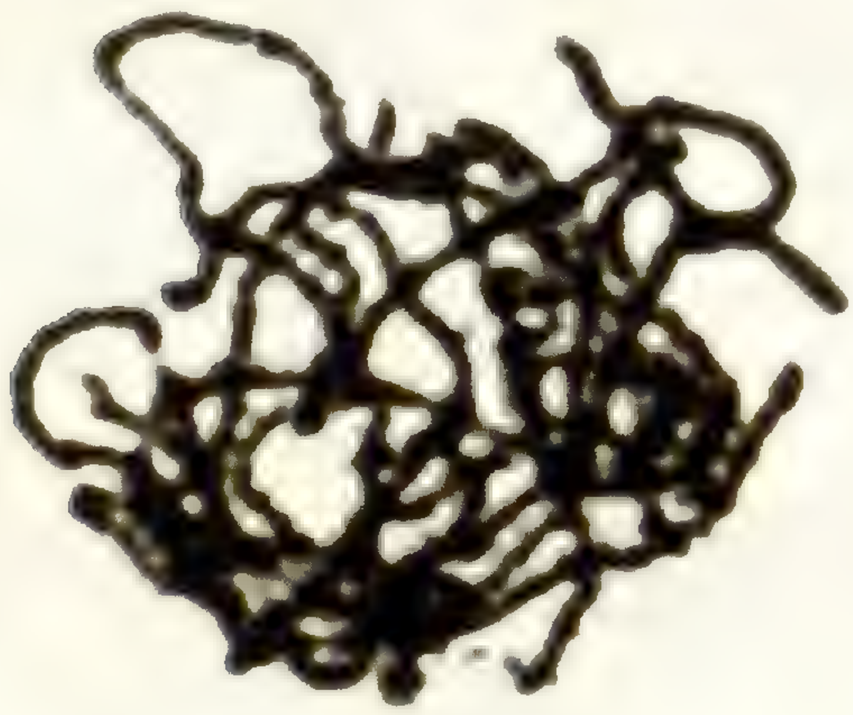

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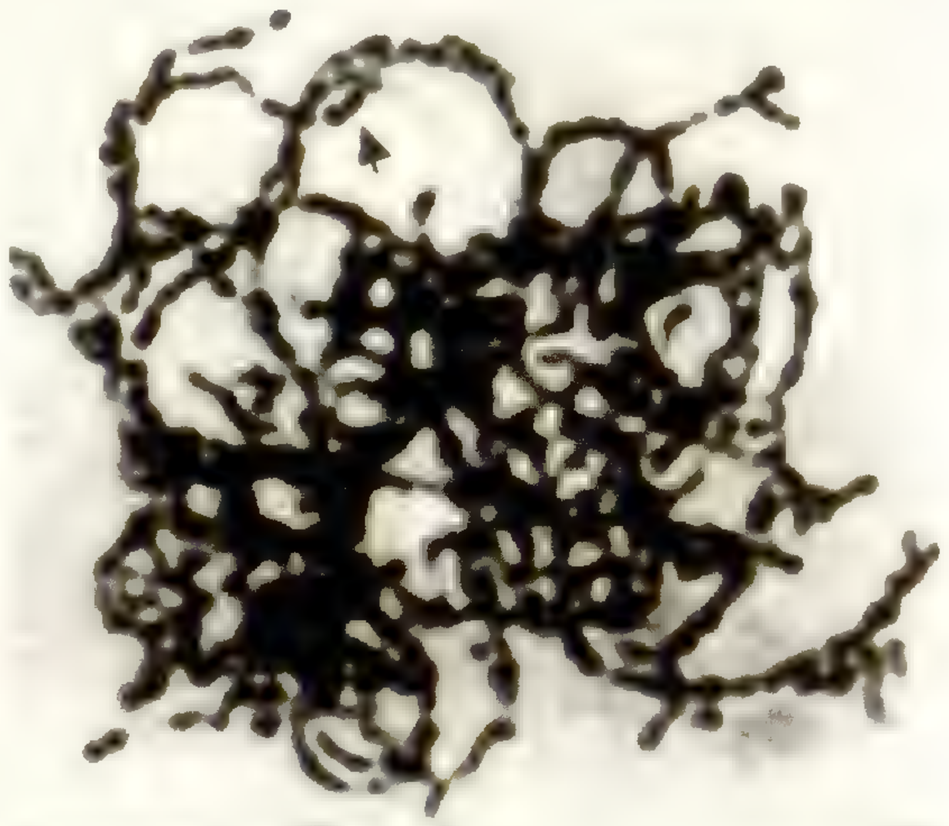

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FIGS. 15–20. Microsporogenesis in *Isoetes sinensis*. 15. Leptotene. 16. Late Leptotene. 17–18. Zygotene, showing an unpaired chromosome region (arrow). 19–20. Pachytene. 19. Early Pachytene. 20. Later Pachytene, showing partial duplicate pairing (arrow head). Scale bars in Figs. 15–17 & 19–20 = 5 μm; Fig. 18 = 10 μm.

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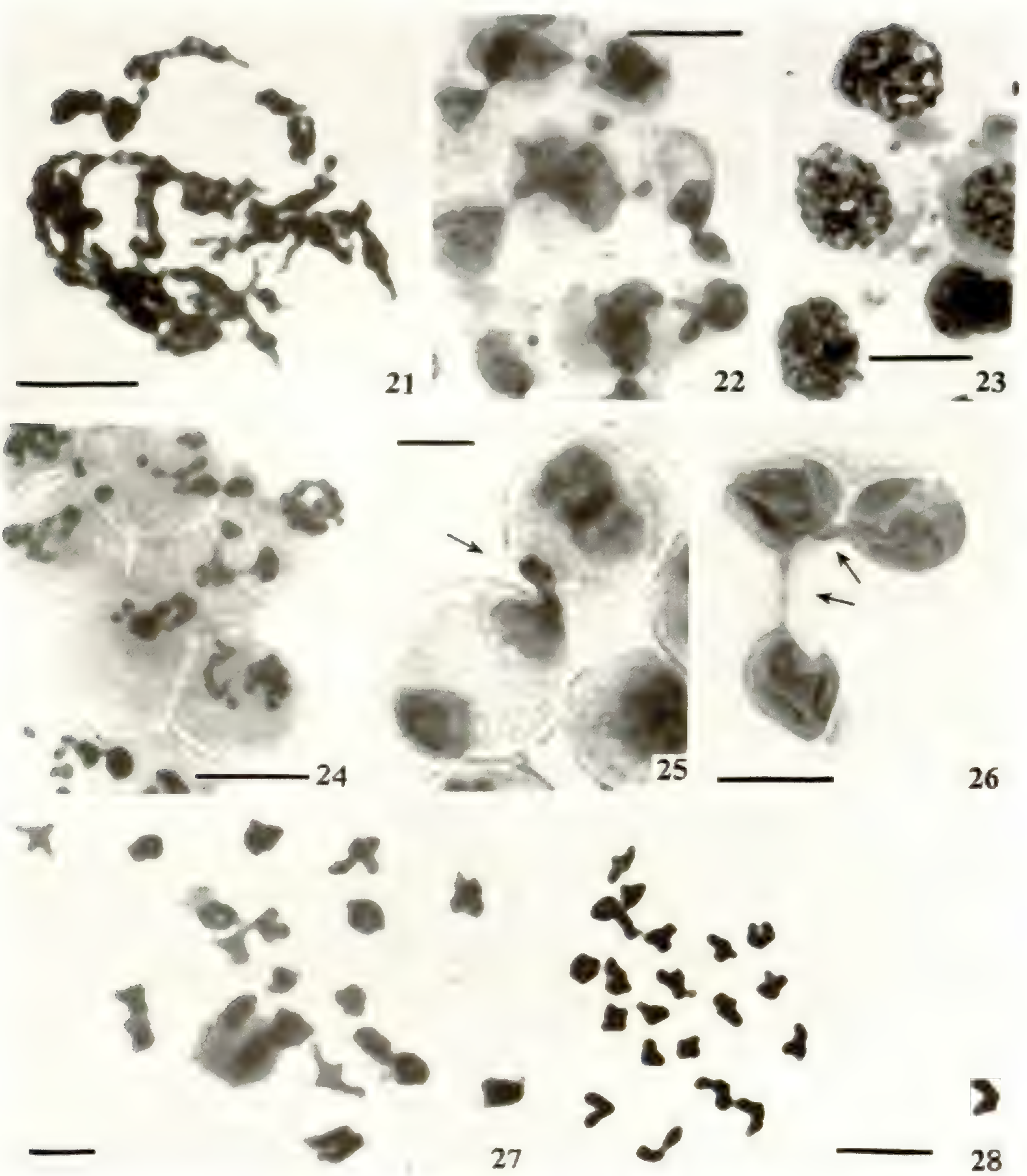


FIGS. 21–28. Microsporogenesis and Megasporogenesis in *Isoetes sinensis*. 21. Late Diplotene. Usually the processes of microsporogenesis in *Isoetes sinensis* from Leptotene to Diplotene are similar to that observed in megasporogenesis. 22–26. Cytomixis of microspore mother cells in meiosis. 22. Cytomixis at Leptotene. 23–24. Cytomixis at Metaphase I. 25. Cytomixis at Telophase I (arrow). 26. Cytomixis at tetrad stage (arrows). 27. Diakinesis in megaspore mother cells. 28. Diakinesis in microspore mother cells. Scale bars in Figs. 27 = 5 μm; Figs. 21, 25 & 28 = 10 μm; Figs. 22–24 & 26 = 20 μm.

multivalents were not seen. In microspore mother cells, secondary synapsis was observed. The configurations formed by these synapses were dependent on the number and position of the chiasmata present in the bivalents. For example, Fig. 28 shows a rhombic bivalent configuration created by two

chiasmata located at the end of the chromosome arms and two at the center of the arms. Nucleoli gradually disappeared during diakinesis.

During metaphase I (Figs. 29–31), all chromosomes arrayed along the metaphase plate. As megaspore mother cells continued to develop and enlarge, the cell wall became thicker and more rigid. In addition, the cytoplasm became filled with metabolic products that obscured the chromosomes and the thicker wall made it difficult to flatten and spread preparations in order to see chromosomes. Therefore, following diakinesis, it was necessary to focus on microspore mother cells to describe cytological changes during the succeeding stages of meiosis. Pairing of bivalents was frequently observed in microspore mother cells (Figs. 29–30). Sometimes two or more bivalents associated to form a short or long chain (Figs. 30–31).

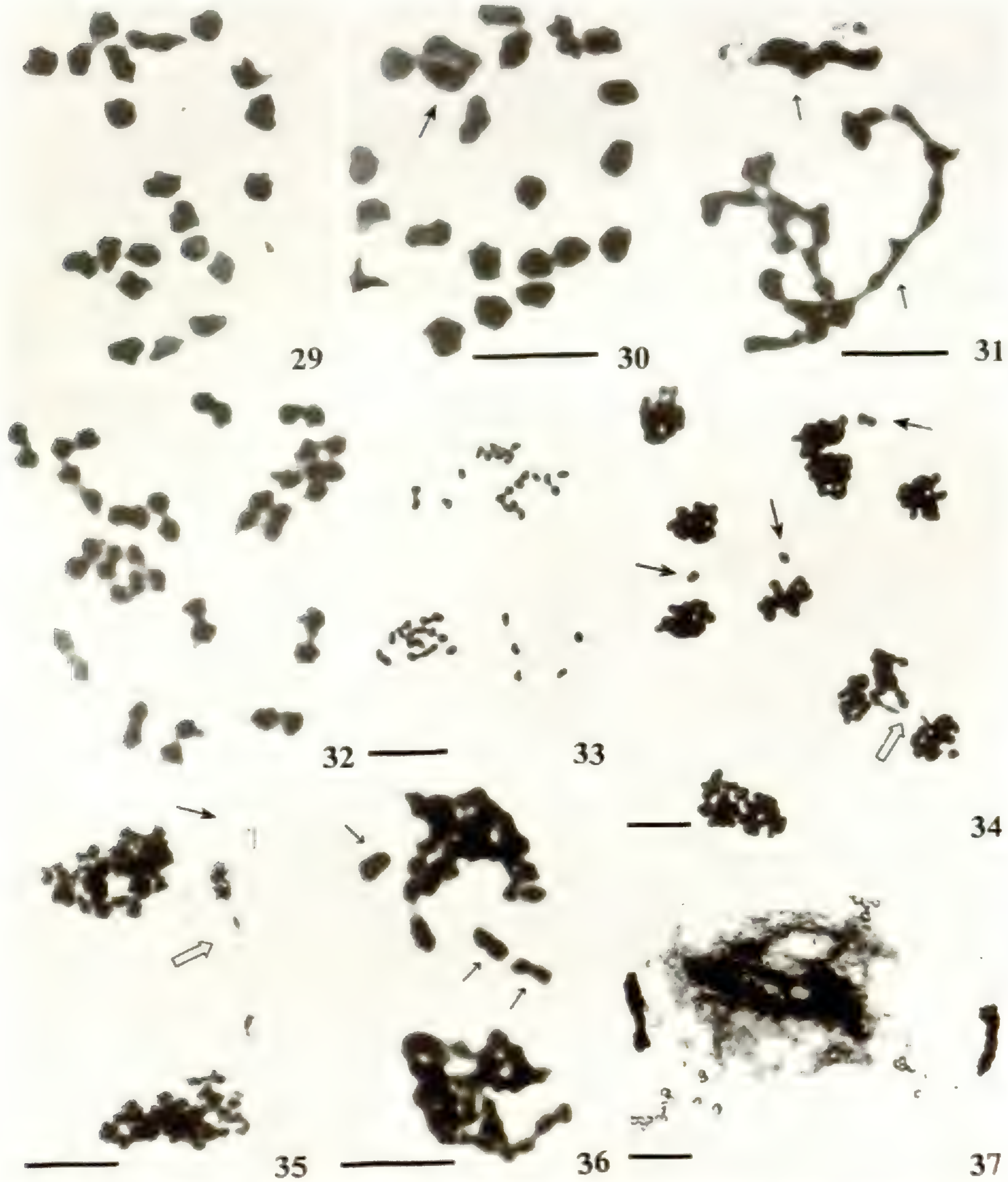
At anaphase I (Figs. 32–33), chromosome configurations in the microspore mother cells were clearly seen. Of the 118 cells evaluated in anaphase I, 24.4% showed abnormalities such as the presence of lagging chromosomes (Figs. 34, 36), chromosome fragments, or chromosome bridges (Figs. 34–35). It was uncertain if there were such abnormalities in megaspore mother cells since the densely stained cytoplasm obscured the chromosomes (Fig. 37). During telophase I, chromosomes uncoiled into chromatin, nucleoli and nuclear membranes reappeared, and dyads formed.

The Second Meiotic Division.—In prophase II, chromatin in the daughter nucleus of each dyad condensed and coiled into chromosomes again. At metaphase II, the centromere of each chromosome split and the chromatids separated. During anaphase II, the four daughter nuclei became arranged as if they were at the ends of a cross. Lagging chromosomes and micronuclei were found in 26.9 % of 191 microspore mother cells observed (Fig. 38). In a few of these cells, unbalanced divisions occurred yielding two larger and two smaller daughter nuclei (Fig. 39). In such case, the resulting tetrad of microspore mother cell daughter nuclei formed a one-dimensional tetragonal configuration. In contrast, the tetrad of the megaspore mother cell daughter nuclei formed a three-dimensional tetrahedral configuration (Figs. 42–43), and the walls became further thickened.

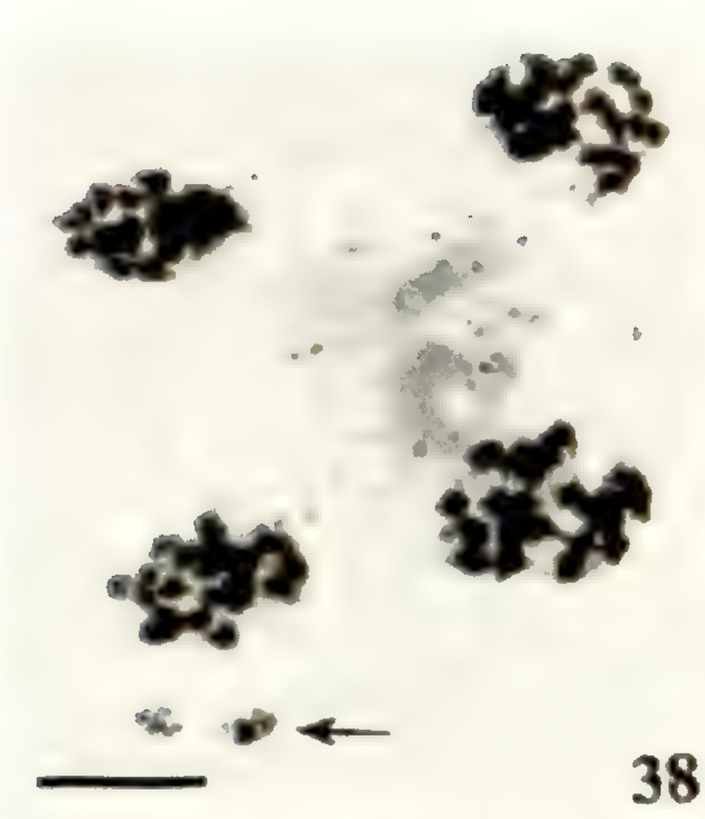
Approx. 8.4 % of 152 randomly counted megaspores appeared to be irregular in size and form (Fig. 44). Approx. 14.8% of 266 microspores were observed to be irregular in size and form (Figs. 40–41). This percentage of irregular microspores was considerably lower than the 24.4% incidence of abnormal chromosomal behavior noted in microspore mother cells during anaphase I (Figs. 34–36).

Discussion

Seven species of *Isoetes* are currently recognized in East Asia (Liu *et al.*, 2002, Takamiya *et al.*, 1997). These include four basic diploid species ($2n = 22$) *I. asiatica* (Makino) Makino, *I. hypsophila*, *I. taiwanensis* and *I. yunguiensis* and three polyploid species *I. sinensis* ($2n = 44$), *I. japonica* A. Braun ($2n = 66$), and *I. pseudojaponica* M. Takamiya, Mitsu Watan. & K. Ono ($2n = 88$). Chung and Choi (1986), described *I. coreana* Y. H. Chung & H. K. Choi ($2n = 66$), but



FIGS. 29–37. Microsporogenesis and Megasporogenesis in *Isoetes sinensis*. 29–31. Metaphase I in microspore mother cells. 29. 22 bivalents. 30–31. Secondary synapsis (arrows). 32–37. Anaphase in microspore mother cells. 32. Early anaphase I, showing sister chromatids together only at their centromeres. 33–37. Anaphase I, 33. Chromosomes at poles. 34. Mother cells, showing a chromosome bridge (hollow arrow) and lagging chromosomes (arrows). 35. Chromosome bridge (hollow arrow) and chromosome fragment (arrow). 36. Lagging chromosomes (arrows). 37. Anaphase I in megaspore mother cells. Scale bars in Fig. 32 = 5 μm; Figs. 29–31, 33 & 35–37 = 10 μm; Fig. 34 = 20 μm.



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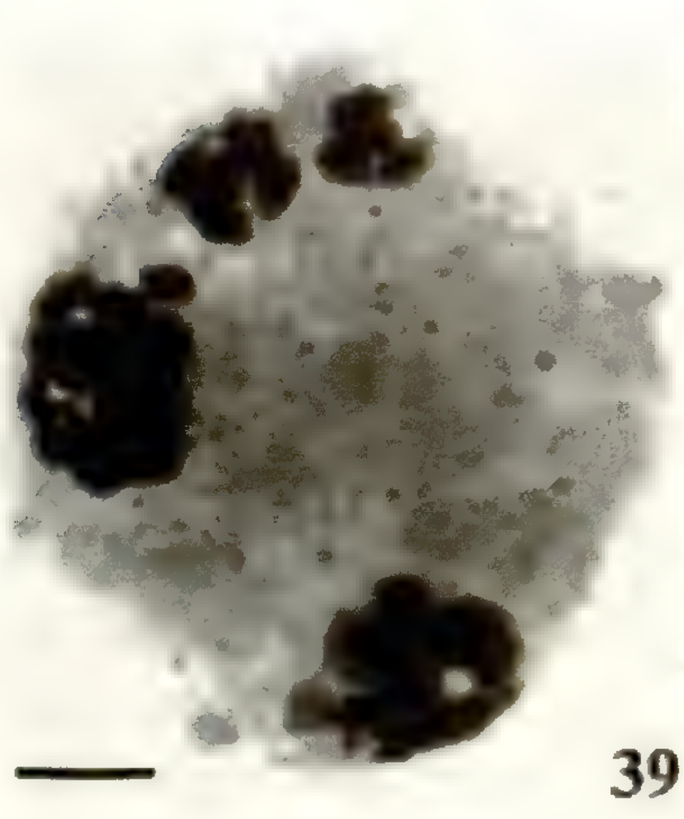

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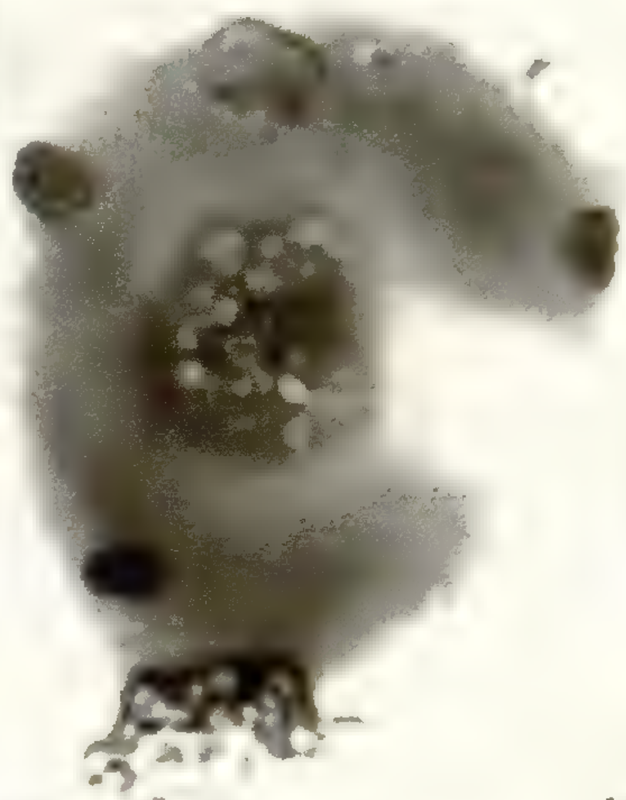

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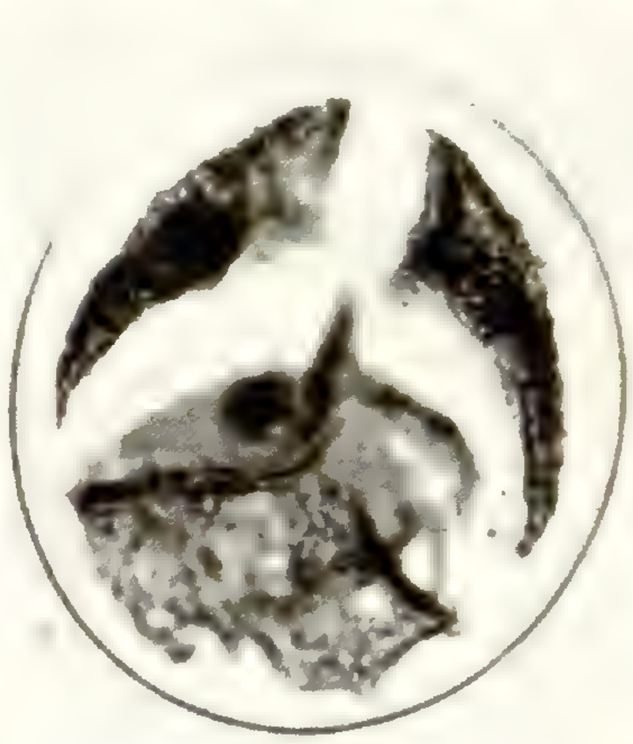

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FIGS. 38–45. Microsporogenesis and Megasporogenesis in *Isoetes sinensis*. 38–39. Anaphase II in microspore mother cells. 38. Lagging chromosomes (arrows). 39. Unbalanced divisions. 40–41. Microspores. 40. Irregular shaped spore (arrow). 41. Small, irregular spores (arrow). 42. Anaphase II in megaspore mother cells. 43. Tetrad of megaspores. 44. Megaspores, irregular megaspores (arrows). 45. Megaspore and tetrads of microspores (arrow) in a mixed sporangium. Scale bar in Figs. 39 = 5 μm; Figs. 38 = 10 μm; Figs. 40–43 = 20 μm; Figs. 44–45 = 40 μm.

Takamiya *et al.* (1997) reduced this hexaploid taxon to *I. sinensis* var. *coreana*. Takamiya *et. al.* (1996) reported on chromosome behavior during meiosis for Japanese species of *Isoetes*. In this paper, we provided a more detailed analysis of chromosome behavior in *I. sinensis* with an emphasis on prophase I chromosome behavior in both megaspore and microspore mother cells.

We observed the mixed sporangia in *Isoetes sinensis*, and this result was consistent with the reports in *I. pantii* Goswami & Arya (Goswami, 1975) and *I. yunguiensis* (Wang *et al.*, 2002). *Isoetes sinensis* showed chromosome pairing and bivalent formation similar to that observed in basic diploid species except that twenty-two bivalents formed in this tetraploid instead of the eleven bivalents observed in basic diploid species. These observations of bivalent formation in *I. sinensis* were consistent with those previously reported for allopolyploids by Takamiya *et al.* (1996).

In *Isoetes sinensis*, megasporogensis appears to be a more reliable process than microsporogensis in the production of uniform meiotic products. During the early stages of megasporogenesis where chromosomes could be clearly observed, chromosome behavior appeared normal. Only about 8% of the megaspores produced appeared to be irregular in size and form. In contrast, during microsporogenesis, microspore mother cells showed secondary pairing of bivalents and abnormal chromosome behavior in nearly 25% of the cells examined during anaphase I. About 15% of the microspores produced appeared to be irregular in size and form.

Cytomixis, the transfer of cytoplasmic and nuclear contents from one cell to another, has been observed in pollen mother cells of angiosperms (Cheng *et al.*, 2001; Malallah and Attia, 2003), but cytomixis has not been reported in *Isoetes*. Cytomixis can lead to the production of abortive pollen grains (Sapare, 1978; Samushia *et al.*, 1979). Drugs or mechanical pressure may cause cytomixis (Bobak and Herich, 1978; Morisset, 1978). However, cytomixis can occur naturally to produce variations in chromosome number (Cheng *et al.*, 1980; Cheng *et al.*, 1982). Whatever its cause, cytomixis in *I. sinensis* was characterized by: (1) an occurrence during all stages of meiosis; (2) a frequency of incidence which varied among individuals, sporangia, and locations in the same sporangium, and (3) the presence of intercellular spaces along the perine of microspore mother cells undergoing cytomixis. Abnormalities observed as a consequence of cytomixis included unbalanced chromosome numbers, micronuclei, and enucleate cells.

We cautiously assert that cytomixis is a natural phenomenon in *I. sinensis*. We do not believe that our experimental methods caused cytomixis since we have used the same methods to study meiosis in members of the Taxodiaceae, Magnoliaceae, Styracaceae, and Actinidiaceae where cytomixis was not always seen.

Isoetes sinensis is an element of the Sino-Japan forest subkingdom (Wu and Wu, 1998). Generally, in *Isoetes*, each megasporangium contained 40–60 megaspore mother cells and each microsporangium contained ca 75, 000 microspore mother cells (Smith, 1900). We do not believe that the incidence of abnormal chromosome behavior we observed in our study could greatly impede the reproduction and dispersal of *I. sinensis*. We have observed that in

native populations and in botanical garden cultures, *I. sinensis* reproduces effectively. *Isoetes sinensis* is known from more than thirteen populations in five Chinese Provinces, but field studies have revealed that eleven of these populations, one in Nanjing, Jiangsu (the type locality), four in Zhejiang, four in Anhui, one in Guangxi, and another in Jiangxi, may no longer exist. Of the remaining four sites, the Songyang and Jiande, (Zhejiang) populations cover less than 1000 m^2 (Ye and Li, 2003), the Xiuning, Anhui population covers less than 900 m^2, and the Guilin (Guangxi) population has only a few individuals remaining. Although cytomixis, lagging chromosomes, chromosome bridges, chromosome fragments, and micronuclei observed during meiosis in *I. sinensis* may have some effect on sexual reproduction, they probably play only a minor factor in population loss. The main cause of population decline of *I. sinensis* appears to be from human disturbance. With increasing urbanization, destruction of wetlands, and water pollution, habitats for *I. sinensis* are becoming degraded and fragmented and, as a result, populations are dwindling. Intensive searches to locate additional native populations as well as strict conservation measures to safeguard the remaining known populations are urgently needed.

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Phylogenetic Relationships of *Isoëtes* (Isoëtaceae) in China as Revealed by Nucleotide Sequences of the Nuclear Ribosomal ITS Region and the Second Intron of a *LEAFY* Homolog

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Abstract.—*Isoëtes* is an ancient lycopod lineage with a highly conserved morphology that provides few morphological characters to resolve the phylogeny of its species. Species appear to have evolved by divergence and allopolyploidy. The basic diploids *I. hypsophila*, *I. taiwanensis*, and *I. yunguiensis* and the tetraploid *I. sinensis* occur in China. Analysis of ITS sequences indicates that the Chinese *Isoëtes* species are part of an Australasian clade including *I. brevicula* from Western Australia and *I. kirkii* from New Zealand. Two distinct cloned sequences of the second intron of a *LEAFY* homolog were recovered from *I. sinensis* supporting the hypothesis that *I. sinensis* is an allotetraploid. One of the *I. sinensis* cloned sequences was similar to the *I. taiwanensis* sequence and the other cloned sequence was similar to the *I. yunguiensis* sequence identifying *I. taiwanensis* and *I. yunguiensis* as the likely parents of *I. sinensis*. Other cloned sequences recovered from *I. sinensis* were recombined parts of the two distinct sequences. Morphological evidence supporting an allotetraploid origin of *I. sinensis* is found in its larger microspore size and intermediate megaspore texture compared to *I. taiwanensis*, and *I. yunguiensis*.

Isoëtes L. is a cosmopolitan genus of heterosporous lycopods containing hundreds of species. Plants usually appear as tufts of linear leaves arising from an underground, corm-like rootstock. Ellipsoidal sporangia occur in expanded leaf bases. Species range from evergreen aquatics to ephemeral terrestrials. Although *Isoëtes* is an ancient lineage with its distinctive morphology recognizable in the Triassic (Retallack, 1997), few characters have been found in its highly conserved morphology to resolve the phylogenetic relationships of its species. Distinguished by their habitat preference, megaspore morphology, and chromosome numbers, species appear to have evolved by ecological isolation and genetic divergence as separated populations adapted to terrestrial or aquatic habitats and by interspecific hybridization and chromosome doubling (allopolyploidy) when divergent species were dispersed into the same sites (Taylor et al., 1993).

Interspecific hybridization and allopolyploidy are well documented for *Isoëtes*. Many interspecific hybrids have been recognized by their production of irregular spores and confirmed by their chromosome numbers (Brunton and

Britton, 1999). In several cases, interspecific hybrids and their suspected allopolyploid derivatives have been verified by chromosome counts, isozyme profiles, and DNA sequences (Taylor and Hickey, 1992; Hoot and Taylor, 2001; Hoot et al., 2004). A polyploid series ranging from $3x = 33$ to $12x = 132$ is known for *Isoëtes*. Over 60% of *Isoëtes* taxa, for which chromosome counts have been published, are polyploid (Troìa, 2001). Therefore, not only is there documentation that interspecific hybridization and allopolyploidy occur in *Isoëtes*, but there is also evidence that allopolyploidy is an equally important mechanism of speciation in this genus.

Recently, herbarium, field, and laboratory studies have been conducted to learn more about the *Isoëtes* of China. These studies have provided an opportunity to determine the status of historical populations, discover new populations and new taxa, and obtain live specimens from which root tips could be harvested for chromosome counts and fresh leaves could be collected for DNA isolations.

Four species of *Isoëtes* have been described for China. All are believed to be rare and endangered. *Isoëtes hypsophila* Handel-Mazzetti is known from the Hengduan Mountains in northwestern Yunnan Province and southwestern Sichuan Province. In this region, *I. hypsophila* occurs in the shallow water of lakes and ponds about 3600 m above sea level. *Isoëtes sinensis* T. C. Palmer has been found at about ten sites in and along rivers and lakes of the middle and lower Yangtze River system. At present, only three populations are known to remain in China. *Isoëtes sinensis* has also been reported from the Kyushu and Chubu Districts Japan (Takamiya et al., 1997) and Cheju Island, South Korea (Takamiya, 2001). *Isoëtes taiwanensis* DeVol is known only from Menghuan Lake near the foot of Zhixing Mountain in the Yangming Mountains National Park, north of Taipei in northern Taiwan. *Isoëtes yunguiensis* Q. F. Wang and W. C. Taylor is known from the Yunnan–Guizhou Plateau in southwest China. In this region, plants have been recorded at four sites, but only two small populations, totaling about 400 individuals, are known to remain. Liu et al. (2002) reported that *I. hypsophila*, *I. taiwanensis*, and *I. yunguiensis* are basic diploids ($2n - 2x = 22$) and *Isoëtes sinensis* is a tetraploid ($2n = 4x = 44$).

Nucleotide sequences from the nuclear ribosomal ITS region, the chloroplast *atpB-rbcL* spacer region, and the second intron of a *LEAFY* homolog have been used to determine phylogenic relationships of *Isoëtes*, delimit species, and reveal an interspecific hybrid and its allotetraploid derivative (Hoot and Taylor, 2001). Hoot *et al.* (2004) used cloning to separate homoeologous sequences of the second intron of a *LEAFY* homolog for several *Isoëtes* allotetraploids. By comparing these cloned sequences to those of putative parents, some of the parent species could be identified.

The goals of this paper were to use nucleotide sequences from the nuclear ribosomal ITS region and the second intron of a *LEAFY* homolog to: (1) determine the relationships of the Chinese *Isoëtes* species, (2) test the hypothesis that the tetraploid *I. sinensis* is an allotetraploid and, if this hypothesis is correct, (3) identify the basic diploid parent species of *I. sinensis*.

TABLE 1. Specimens sampled. Columns indicate species, location, collector, collection number-DNA isolation number, date of collection, and herbarium acronym for location of voucher. Collections are from Mainland China unless otherwise noted.

Species	Voucher Collection	Isolation Number
Isoëtes brevicula	Rock pool, summit of Lily McCarthy Rock, Western Australia, 25 Sep 2002, *W. C. Taylor & N. T. Luebke 6383* (MIL)	189
Isoëtes hypsophila	Tu-er-sjan, Dao-cheng County, Sichuan Province, 01 Aug 2001, *Wang Aing-Feng, Liu Xing, Liu Hong & Yang Xiao-Lin 2* (WH)	127
Isoëtes kirkii	Lake Brunner, South Island, New Zealand, 27 Mar 2004, *D. W. Woodland & Felicity Cutten s.n.* (MIL)	*kiNZ*
Isoëtes sinensis	Xing-an-jiang, Jiande City, Zhejiang Province, 19 Oct 2001, *Liu Xing & Pang Xin-An 3, 4* (WH)	129, 131
Isoëtes taiwanensis	Menghuan Lake, Yangming Mountains, Taiwan, May 1998, *Chiou Wen-Liang s.n.* (MIL)	78
Isoëtes yunguiensis	Sha-shi-chong, Ping-ba County, Guizhou Province, 15 Aug 2001, *Liu Xing & Yang Xiao-Lin 5* (WH)	130

MATERIALS AND METHODS

Species sampling.—Table 1 contains locality, collector, collection number, and location of voucher specimens for plants used in this study. Specimens were identified to species using the original descriptions of the species (Handel-Mazzetti, 1923; Palmer, 1927; DeVol 1972a; Wang Q. F. *et al.*, 2002) and by comparison with authentic and type specimens. Diagnostic morphology for megaspore textures was evaluated using an Olympus SZX12 stereomicroscope.

DNA isolation and amplification.—DNA was isolated from 20 mg of silica dried leaves from each sample by grinding the leaf tissue, frozen in liquid nitrogen, to a powder with a disposable 1.5 pellet pestle (Kimble-Kontes) in a 1.5 ml snap-cap microcentrifuge tube (Eppendorf) and using the DNeasy® Plant Mini Kit (Qiagen) following the manufacturer's protocol.

The ITS region for all samples was amplified with the primers ITS-I (5′-GTCCACTGAACCTTATCATTTAG-3′; Urbatsch, *et al.* 2000) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; White *et al.* 1990). The *LEAFY* intron for all samples was amplified with the primers 30F (5′- GATCTTTATGAA-CAATGTGG-3′) and 1190R (5′- GAAATACCTGATTTGTAACC-3′); Nancy S. Napier designed both *LEAFY* primers. PCR reaction mixtures followed manufacturer protocols using Ready-To-Go™ PCR Beads (Amersham Bio-sciences). PCR amplification for the ITS region began with denaturation for 60 s at 97°C followed by 40 cycles of denaturation for 10 s at 97°C, annealing for 30 s at 48°C, and extension for 20 s at 72°C with 4 s added to extension time each cycle and ending with a final extension of 7 min at 72°C. PCR amplification for the *LEAFY* intron began with denaturation for 5 min at 94°C followed by 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C, and extension for 1 min at 72°C, and ending with a final extension of 5

min at 72°C. PCR products were concentrated via electrophoresis in a 2% agarose gel containing ethidium bromide and visualized with transilluminated UV. Bands were cut from the gel and purified using the QIAquick® Gel Extraction Kit (Qiagen).

Cloning and sequencing.—Sequencing of the ITS PCR products was performed on both 5′ and 3′ DNA strands using the amplification primers ITS-I and ITS4 as cited above.

Ligation of the purified *LEAFY* PCR product and subsequent transformation, cloning, and visualization of transformed clones followed manufacturer protocols using the pGEM®-T Easy Vector Systems (Promega) with LB Amp 100 X-gal plates (Teknova).

For basic diploid species, *I. taiwanensis* and *I. yunguiensis*, eight clones (colonies) for each species were sequenced. For the tetraploid species, *I. sinensis*, 16 clones (eight from each of two plants) were sequenced to increase the odds of capturing all parental cloned sequences. For the outgroup species, *I. brevicula* E. R. L. Johnson, *I. hypsophila*, and *I. kirkii* A. Braun, four clones from each species were sequenced.

Cleaning and concentration of the vector DNA followed manufacturer protocols using the QIAPrep® Spin Miniprep Kit (Qiagen). Sequencing was performed on both 5′ and 3′ DNA strands using sequencing primers M13F and M13R in conjunction with the ABI Big Dye® Terminator Cycle v 3.1 Sequencing Kit (Applied Biosystems) following the manufacturer's protocol. Sequencing products were resolved on an ABI (model 377) DNA sequencer at the Iowa State University DNA Sequencing and Synthesis Facility, Ames, Iowa.

Sequence alignment and phylogenetic analysis.—Nucleotide sequences were aligned and edited using Sequencher 4.1 (Gene Codes Corp.). Gaps were treated as additional presence/absence characters, with one or multiple base gaps scored as a single character (Baldwin *et al.* 1995).

Maximum parsimony analysis of the data was conducted with PAUP* version 4.0b10 (Swofford, 2002) using the heuristic search option for the ITS data set and the *LEAFY* data set, maximum trees = 4000 for the ITS data set and maximum trees = 100 for the *LEAFY* data set. PAUP* was used to run 500 bootstrap replicates for each data set to estimate reliability of the clades (Felsenstein, 1995).

RESULTS

ITS sequences of *Isoëtes hypsophila*, *I. taiwanensis* and *I. yunguiensis* from China, *I. brevicula* from southwestern Australia, and *I. kirkii* from New Zealand form an Australasian clade with other species and clades previously reported by Hoot and Taylor (2001). The data set analyzed consisted 16 ingroup species and two outgroup species with a total of 826 characters; 313 characters were variable and 239 were parsimony informative. The ITS tree illustrated is a bootstrap 50% majority-rule consensus tree of the 39 most parsimonious trees retained (Fig. 1). Tree topology shows a close relationship

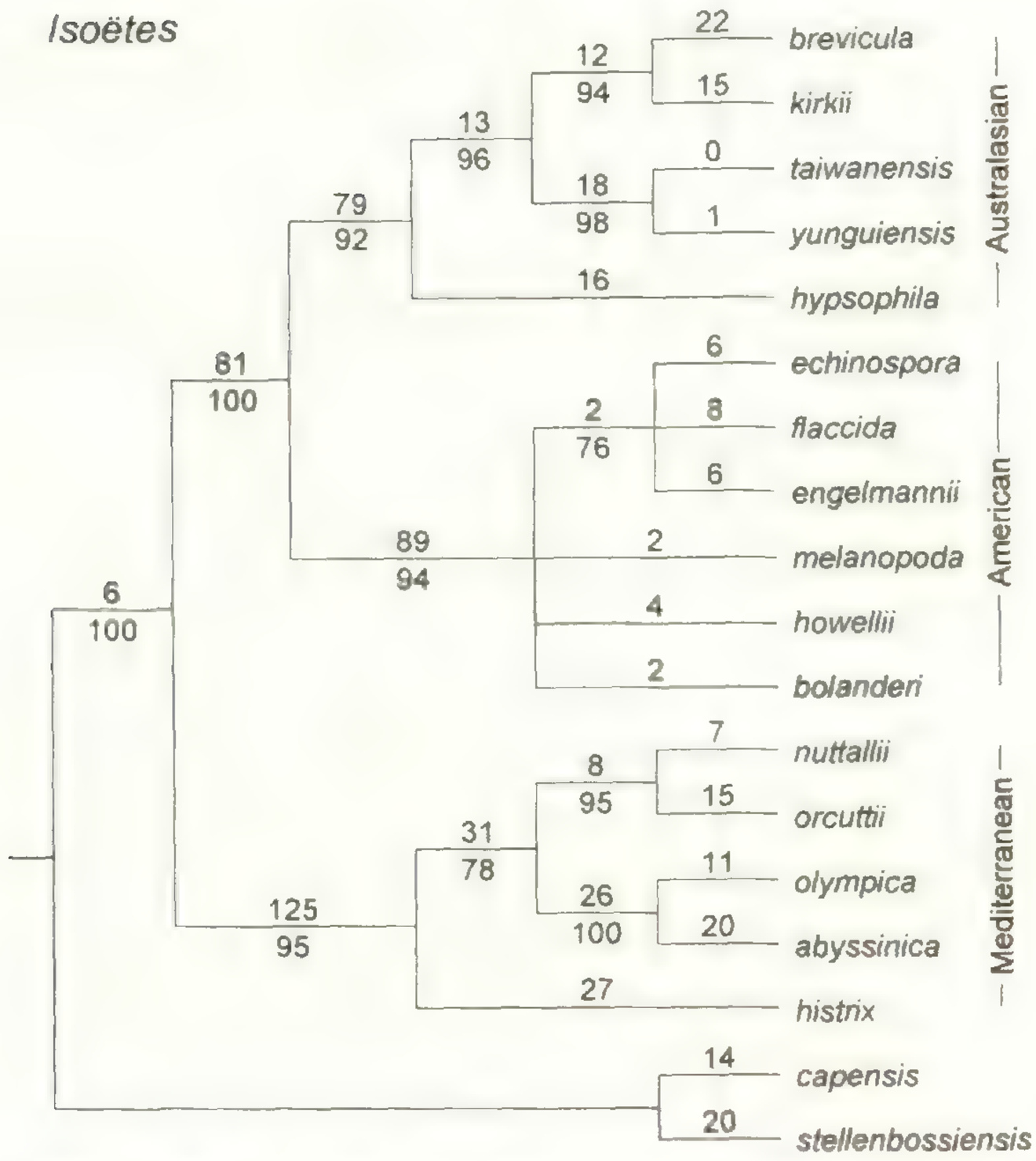

FIG. 1. *Isoëtes* ITS region tree. Bootstrap 50% majority-rule consensus tree of 39 trees resulting from maximum parsimony analysis using heuristic search of ITS region sequence data for eighteen basic diploid species of *Isoëtes*. Tree length is 489 steps of equally weighted nucleotide substitutions and gaps, CI (excluding uninformative characters) = 0.76, RI = 0.86. Numbers above the branches are the number of nucleotide substitutions. Numbers below the branches are bootstrap values. Major clades are labeled on the right. The tree is rooted with *I. capensis* and *I. stellenbossiensis* from South Africa.

exists between *I. taiwanensis*, and *I. yunguiensis*. All species in the tree are basic diploids ($2n = 22$).

Defined by the 30F and 1190R Napier primers, the *LEAFY* region for aligned sequence clones of *I. sinensis*, *I. taiwanensis*, and *I. yunguiensis* was 1105 bases long and included all of the second intron and parts of the flanking exons. All of the eight sequence clones of *I. taiwanensis* were 1075 bases long and all of the eight sequence clones of *I. yunguiensis* were 1072 bases long. Two of the 16 *I. sinensis* clones did not amplify for the *LEAFY* intron. Therefore, 14 aligned, cloned sequences were compared for informative sites. At 15 informative sites that included 12 substitutions, two one base gaps, and one three base gap, six cloned sequences of *I. sinensis*, each 1076 bases long, matched or nearly matched the cloned sequence type of *I. taiwanensis* and five cloned sequences of *I. sinensis*, each 1079 bases long, matched or nearly matched the consensus cloned sequence type of *I. yunguiensis* (Table 2). Seven *I. sinensis* cloned sequences showed evidence of recombination i.e., part of the PCR amplified, cloned sequence first matched either the *I. taiwanensis* or the *I. yunguiensis* sequence type, but further on matched the other sequence type.

TABLE 2. Comparison of fourteen cloned sequences of *I. sinensis* with cloned sequences of *I. taiwanensis* and *I. yunguiensis* at fifteen informative sites. Sites are numbered sequentially beginning with the 30F Napier primer. *Isoëtes sinensis* cloned sequences are identified by collection number-DNA isolation number-clone number. Nucleotides in italic compare to those of *I. taiwanensis*. Nucleotides in bold face compare to those of *I. yunguiensis*. Gaps are indicated by dashes. Sequences marked with an asterisk showed extensive recombination and were removed from the data set before the final analysis. Clones 4-131-6 and 4-131-7 did not amplify for the *LEAFY* region and are not included in the table.

site/clone	205	289	290	357	525	549	660	741	776	827	840	865	965-967	987	1059
taiwanensis	*C*	*G*	*T*	*T*	*C*	*G*	*T*	*T*	*A*	*A*	*A*	*A*	- - -	-	*A*
3-129-3	*C*	*G*	*T*	*T*	*C*	*G*	*T*	*T*	*A*	*A*	*A*	*A*	- - -	-	*A*
3-129-7	*C*	*G*	*T*	*T*	*C*	*G*	*T*	*T*	*A*	*A*	*A*	*A*	- - -	-	*A*
4-131-1	*C*	*G*	*T*	*T*	*C*	*G*	*T*	*T*	*A*	*A*	*A*	*A*	- - -	-	*A*
4-131-3	*C*	*G*	*T*	*T*	*C*	*G*	*T*	*T*	*A*	*A*	*A*	*A*	- - -	-	*A*
4-131-4	*C*	*G*	*T*	*T*	*C*	*G*	*T*	*T*	*A*	*A*	*A*	*A*	- - -	-	*A*
3-129-5	*C*	*G*	*T*	*T*	*C*	*G*	*T*	**C**	*A*	*A*	*A*	*A*	- - -	-	**G**
3-129-2*	*C*	*G*	*T*	*T*	**T**	**T**	**C**	**C**	**G**	**G**	-	**G**	- - -	-	*A*
3-129-1*	*C*	*G*	*T*	*T*	**T**	**T**	**C**	**C**	**G**	**G**	-	**G**	**A T G**	**T**	*A*
4-131-8*	**T**	**A**	**C**	**C**	*C*	*G*	*T*	*T*	*A*	*A*	*A*	*A*	- - -	-	*A*
4-131-2	*C*	**A**	**C**	**C**	**T**	**T**	**C**	**C**	**G**	**G**	-	**G**	**A T G**	-	**G**
3-129-4	**T**	**A**	**C**	**C**	**T**	**T**	**C**	**C**	**G**	**G**	-	**G**	**A T G**	-	**G**
3-129-8	**T**	**A**	**C**	**C**	**T**	**T**	**C**	**C**	**G**	**G**	-	**G**	**A T G**	-	**G**
3-129-6	**T**	**A**	**C**	**C**	**T**	**T**	**C**	**C**	**G**	**G**	-	**G**	**A T G**	**T**	**G**
4-131-5	**T**	**A**	**C**	**C**	**T**	**T**	**C**	**C**	**G**	**G**	-	**G**	**A T G**	**T**	**G**
yunguiensis	**T**	**A**	**C**	**C**	**T**	**T**	**C**	**C**	**G**	**G**	-	**G**	**A T G**	**T**	**G**

Three sequences showing evidence of extensive recombination were removed from the data set evaluated by PAUP*.

Based on the relationships indicated in the ITS tree (Fig. 1), *I. brevicula*, *I. kirkii*, and *I. hypsophila* were chosen as outgroup species to root the *LEAFY* second intron tree. The sequenced *LEAFY* region for *I. brevicula* was 1035 bases, for *I. kirkii* it was 1090 bases, and for *I. hypsophila* it was 1095 bases.

The *LEAFY* second intron data set analyzed contained 16 sequences with a total of 1125 characters; 171 characters were variable and 54 were parsimony informative. The data set included consensus sequences from clones of *I. brevicula*, *I. hypsophila*, *I. kirkii*, *I. taiwanensis*, and *I. yunguiensis* and 11 cloned sequences of *I. sinensis*. The *LEAFY* second intron tree illustrated is a bootstrap 50% majority-rule consensus tree of the two most parsimonious trees retained (Fig. 2). Six of the *I. sinensis* cloned sequences formed a clade with *I. taiwanensis* and five of the *I. sinensis* cloned sequences formed a clade with *I. yunguiensis*.

DISCUSSION

Although *Isoëtes* is Paleozoic in origin (Pigg, 1992), worldwide in distribution, and over time, undoubtedly adapted to changing climates and aquatic to terrestrial habitats on every continent many times, the morphology of *Isoëtes* has been remarkably conserved. Thus, morphology provides few characters that can be used to reliably reconstruct phylogenetic relationships. Nevertheless, pteridologists have speculated about the relationships of *Isoëtes*

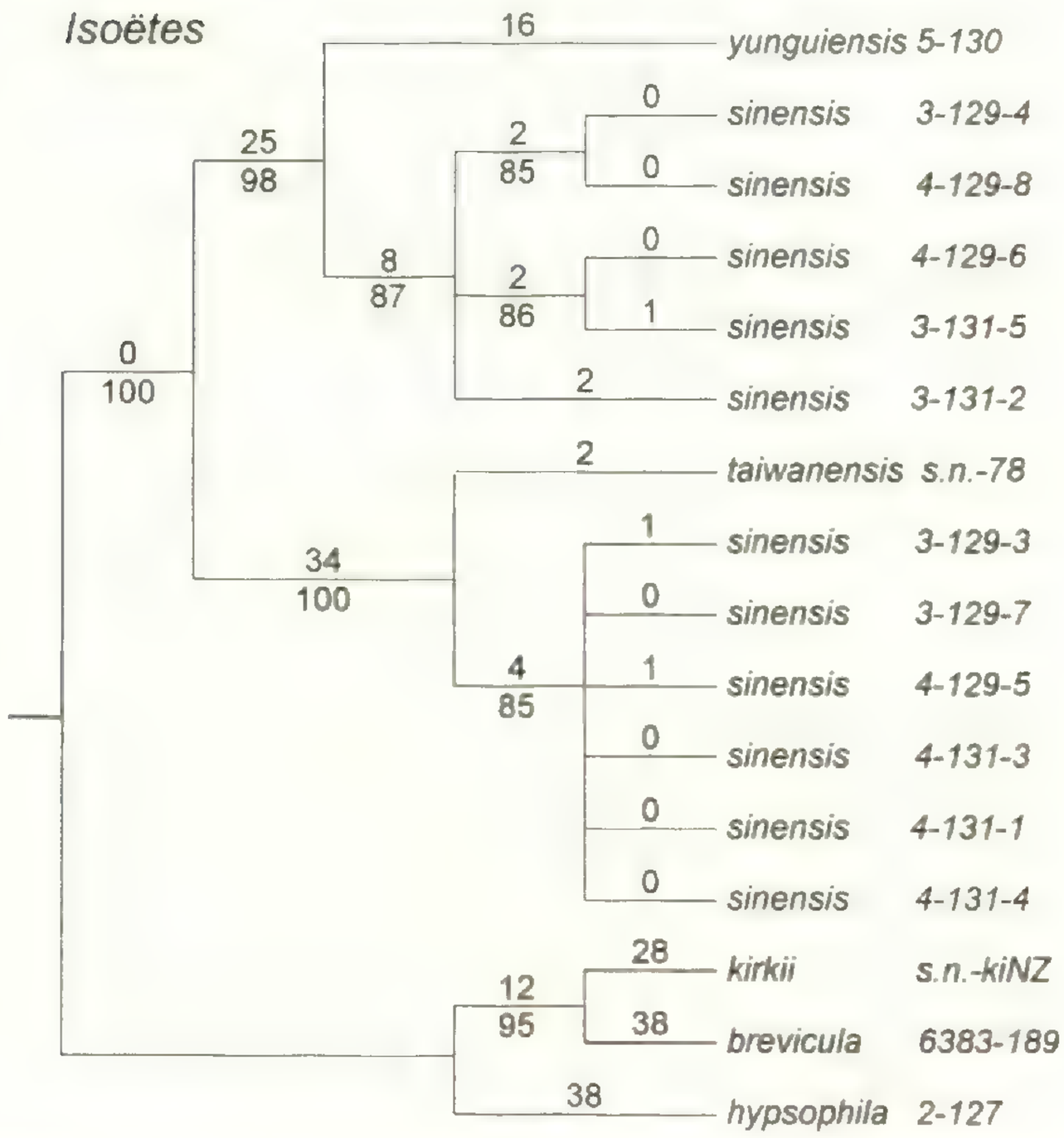

FIG. 2. *Isoëtes LEAFY* second intron homolog tree. Bootstrap 50% majority-rule consensus tree of four trees resulting from maximum parsimony analysis using heuristic search of *LEAFY* second intron homolog sequence data for five basic diploid and one tetraploid (*I. sinensis*) species of *Isoetes*. Eleven sequence clones that form a sister clade to either *I. yunguiensis* or *I. taiwanensis* represent the tetraploid genome of *I. sinensis*. Tree length is 183 steps of equally weighted nucleotide substitutions and gaps, CI (excluding uninformative characters) = 0.89, RI = 0.96. Numbers above the branches are the number of nucleotide substitutions. Numbers below the branches are bootstrap values. Figures to the right of the specific epithets are the collection and clone identification labels. The tree is rooted with *I. hypsophila* from China, *I. brevicula* from Western Australia, and *I. kirkii* from New Zealand.

species based on ecology, morphology, and biogeography. Britton and Brunton (1991) reevaluated the spore morphology of *I. taiwanensis*, concluding that it was not related to taxa from southwestern Australia as proposed by Marsden (1979), but instead appeared to have its closest affinity to *I. kirkii* from New Zealand. The ITS tree (Fig. 1) shows that both *I. brevicula* from southwestern Australia and *I. kirkii* form a sister clade to the Chinese species and all are members of an Australasian clade. Based on spore morphology, habit, and habitat, Huang *et al.* (1992) concluded that *I. taiwanensis* is probably closer to *I. asiatica* than it is to *I. sinensis*, but *I. asiatica* (*I. echinospora* subsp. *asiatica* (Makino) Á. Löve is a member of the *I. echinospora* species complex, a group of circumpolar taxa with echinate megaspores (Löve, 1962, Takamiya, 1997). The ITS tree (Fig. 2) shows that *I. echinospora*, a member of an American clade, is only distantly related to members of the Australasian clade, which includes *I. taiwanensis*. DeVol (1972b) mentioned that *I. taiwanensis* seemed nearer to *I. sinensis* than any other species. Takamiya (2001) saw similarities in the spore morphology of *I. taiwanensis* and *I. sinensis* and concluded that phylogenetic comparisons of these two taxa were needed.

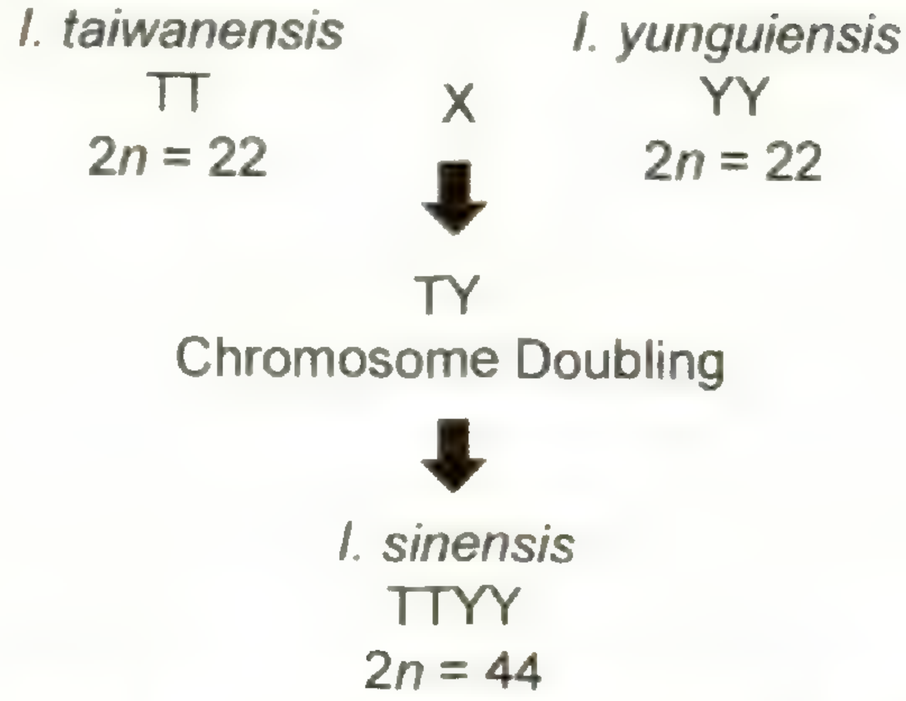

FIG. 3. Hypothetical phylogeny of *Isoëtes sinensis* involving interspecific hybridization and chromosome doubling of the basic diploids *I. taiwanensis* and *I. yunguiensis*. Data presented in this study supports the hypothesis of an allotetraploid origin for *I. sinensis*.

The recovery of two, distinct, *LEAFY* second intron sequence types from the tetraploid *I. sinensis* supports the hypothesis that *I. sinensis* is an allotetraploid (Table 2). To clarify the results, it was assumed that some of the sequenced clones recovered from *I. sinensis* were recombinations of the two distinct sequence types and three of the recombinant sequences were removed from the data set evaluated by PAUP*. Since recombination occurs from crossing over between chromosomes during meiosis, it is possible that the observed recombined sequences were products of natural events. If the recombined sequences detected were the result of crossing-over during meiosis, we would predict that identical crossover sequences would be recovered as clones. All seven of the recombinant cloned sequences from *I. sinensis* were different, indicating that these recombined sequences more likely occurred during the PCR amplification reaction. Whatever their source, including recombinant sequences in a cladistic analysis will affect results and therefore, they need to be recognized and removed from the data set before the final analysis.

Comparison of the two distinct cloned sequences from the allotetraploid *I. sinensis* with the cloned sequences of *I. taiwanensis* and *I. yunguiensis* indicates that either these two basic diploid species, or closely related taxa, likely participated in the formation of *I. sinensis* (Table 2). Although the *I. yunguiensis* clade, including five *I. sinensis* cloned sequences, and the *I. taiwanensis* clade, including six *I. sinensis* cloned sequences, are both well supported with high bootstrap percentages, the *I. yunguiensis* sequence is distinguished from its sister *I. sinensis* clones by sixteen autapomorphies and the *I. taiwanensis* sequence is distinguished from its sister *I. sinensis* clones by two autapomorphies (Fig. 2). These unique nucleotide substitutions could be due to (1) sequencing nucleotides of taxa different from those of the parent taxa, (2) continued evolution of the progenitor parent species and the allotetraploid species following allopolyploidy, or (3) copy errors during PCR. Causes for the autapomorphies might be determined by additional sampling and repeated PCR of the same clones.

In addition to the molecular characters, morphological characters indicate that *I. sinensis*, *I. taiwanensis*, and *I. yunguiensis* are distinct, but closely

related species and provide some evidence supporting the allopolyploid origin of *I. sinensis*. All three species are amphibious plants with tri-lobed rootstocks. They all have a rudimentary velum that covers only the upper edge of the sporangium. The microspores of *I. taiwanensis* and *I. yunguiensis* range from 20–26 μm in length whereas, those of *I. sinensis* range from 26–30 μm in length (Britton and Brunton, 1991; Wang *et al.*, 2002; Palmer, 1927). The larger size of *I. sinensis* microspores is attributed to its increased chromosome number. Increases in chromosome number are usually accompanied by larger spore size (Kott and Britton, 1983). In contrast, megaspores of the tetraploid *I. sinensis* and the basic diploid *I. yunguiensis* average about 400 μm in diameter, whereas megaspores of the basic diploid *I. taiwanensis* average about 300 μm in diameter. Megaspore texture appears to be the most distinctive character that separates these three species. However, in view of the results presented here, the cristate to verrucate megaspores of *I. sinensis* can also be interpreted as subtly combining the textures of the rugulate to reticulate megaspores of *I. taiwanensis* and the cristate to reticulate megaspores of *I. yunguiensis*.

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APPENDIX

Genbank accession numbers for the new *Isoëtes* DNA sequences used in this manuscript.

Species	Genbank accession number
Nuclear ribosomal ITS region sequences	
I. brevicula	AY641098
I. hypsophila	AY641099
I. kirkii	AY641100
I. taiwanensis	AY641101
I. yunguiensis	AY641102
LEAFY second intron homolog sequences	
I. brevicula	AY641103
I. hypsophila	AY641104
I. kirkii	AY641105
I. taiwanensis	AY641106
I. yunguiensis	AY641107
I. sinensis (*I. yunguiensis* type clone)	AY641108
I. sinensis (*I. taiwanensis* type clone)	AY641109

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Authors should adhere to the following guidelines; manuscripts not so prepared may be returned for revision prior to review. Submit manuscripts in **triplicate** (xerox copies acceptable), including review copies of illustrations and originals of illustrations. After review, submission of final versions of manuscripts on diskette (in PC- or Mac-compatible formats) is strongly encouraged. Use standard 8½ by 11 inch paper of good quality, not "erasable" paper. **Double space manuscripts throughout**, including title, authors' names and addresses, short, informative abstract, text (including heads and keys), literature cited, tables (separate from text), and figure captions (grouped as consecutive paragraphs separate from figures). Arrange parts of manuscript in order just given. Include author's name and page number in upper right corner of every sheet. Provide margins of at least 25 mm all around on typed pages. Do not submit right-justified copy, avoid footnotes, and do not break words at ends of lines. Make table headings and figure captions self-explanatory. Use S.I. (metric) units for all measures (e.g., distance, elevation, weight) unless quoted or cited from another source (e.g., specimen citations). For nomenclatural matter (i.e., synonymy and typification), use one paragraph per basionym (see Regnum Veg. 58:39–40. 1968). Abbreviate titles of serial publications according to *Botanico-Periodicum-Huntianum* (Lawrence et al., 1968, Hunt Botanical Library, Pittsburgh) and its supplement (1991). References cited only as part of nomenclatural matter are not included in literature cited. For shorter notes and reviews, omit the abstract and put all references parenthetically in text. Use *Index Herbariorum* (Regnum Veg. 120:1–693. 1990) for designations of herbaria.

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